The Role of $P2Y_{12}$ Receptor Antagonists in Thrombogenesis

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1 Zusammenfassung

1.1 Hintergrund

Der Adenosindiphosphat-Rezeptor $P2Y_{12}$ bewirkt Plättchenaggregation und trägt zur Bildung arterieller Thrombosen bei. $P2Y_{12}$-Antagonisten, wie Clopidogrel und Ticagrelor, werden daher zur Prävention von Herzinfarkten und Schlaganfällen in Patienten mit einem akuten Koronarsyndrom (AKS) verwendet. Ticagrelor war Clopidogrel in der Reduktion der Mortalität in diesen Patienten überlegen und die zugrunde liegenden Mechanismen sind nicht vollständig geklärt. Die arterielle Thrombose ist das zentrale Ereignis in der Entwicklung des AKS und das Gefässendothel ist wesentlich an dessen Entstehung beteiligt. Mögliche Effekte von $P2Y_{12}$-Antagonisten auf das Endothel und seinen prokoagulatorischen Faktoren, insbesondere dem Gewebefaktor, wurden bisher nicht untersucht. Patienten mit AKS haben häufig Komorbiditäten wie Vorhofflimmern (VHF) und benötigen daher eine zusätzliche antikoagulatorische Therapie zum Schutz vor Embolien kardialer Thromben aus dem linken Herzohr. Ähnlich dem Endothel, führt eine Aktivierung des Endokards zu einer Überexpression des Gewebefaktors sowie des Plasminogenaktivator-inhibitors-1, welche zur Thrombogenität in Patienten mit Vorhofflimmern beitragen. Ob bestimmte $P2Y_{12}$-Antagonisten antithrombotische Eigenschaften auf das Endokard linker Herzohren aufweisen und dadurch die Thrombogenität in Patienten mit VHF reduzieren könnten, wurde bisher nicht untersucht.

1.2 Material und Methoden

Menschliche aortale Endothelzellen wurden mit Ticagrelor oder mit dem aktiven Metaboliten von Clopidogrel behandelt und mit Tumor-Nekrose-Faktor-alpha (TNF-α) stimuliert. Anschliessend wurden die Expression und Aktivität des Gewebefaktors sowie die zugrunde liegenden molekularen Mechanismen erforscht. Zusätzlich wurden C57BL/6 Mäuse mit Ticagrelor oder Clopidogrel behandelt und die Expression des endothelialen Gewebefaktors sowie die Bildung arterieller Thrombosen nach photochemische Schädigung des Endothels

der Halsschlagadern untersucht. Schliesslich gewannen wir Endokardzellen aus linken Herzohren von 14 Patienten mit VHF, welche sich einer elektiven Herzoperation unterzogen. Diese wurden mit Ticagrelor oder mit dem aktiven Metaboliten von Clipidogrel behandelt und mit TNF-α stimuliert bevor die Expression und Aktivität des Gewebefaktors und des Plasminogenaktivator-inhibitors-1 analysiert wurden.

1.3 Ergebnisse

Ticagrelor, im Gegensatz zum aktiven Metaboliten von Clopidogrel, verringerte die TNF-α-induzierte Expression und Aktivität des Gewebefaktors durch proteasomale Degradierung unter Einbezug der Signalmoleküle Phosphoinositide-3-Kinase und p70s6 Kinase und unabhängig vom $P2Y_{12}$ Rezeptor sowie dem equilibrative nucleoside transporter 1 (ENT1). Wie in unseren *in vitro* Experimenten, reduzierte Ticagrelor, nicht aber Clopidogrel, die Expression des endothelialen Gewebefaktors in Mausarterien und verlängerte die Zeit bis zur Entstehung arterieller Thrombosen. Dabei waren die Plättchenhemmung, die Gewebefaktoraktivität im Plasma und die systemische Koagulation in beiden Gruppen vergleichbar. In Endokardzellen linker Herzohren von Patienten mit Vorhofflimmern verminderte Ticagrelor, nicht aber Clopidogrel, die TNF-α-induzierte Expression und Aktivität des Gewebefaktors und des Plasminogenaktivator-inhibitors-1.

1.4 Konklusionen

Ticagrelor, nicht aber Clopidogrel, weist lokale antithrombotische Eigenschaften auf das Endothel auf und reduziert die Entstehung von arteriellen Thrombosen im Vergleich zu Clopidogrel. Zudem zeigt Ticagrelor, nicht aber Clopidogrel, lokale antithrombotische Effekte im Endokard linker Herzohren von Patienten mit VHF auf. Die antithrombotischen Eigenschaften von Ticagrelor tragen möglicherweise zur Reduktion der Mortalität von Patienten mit AKS in klinischen Studien bei und vermindern allenfalls das Risiko für systemische Thromboembolien in Patienten mit Vorhofflimmern.

2 Summary

2.1 Background

The adenosine diphosphate receptor $P2Y_{12}$ mediates platelet aggregation and contributes to arterial thrombus formation; therefore, $P2Y_{12}$ antagonists, such as clopidogrel and ticagrelor, are used to prevent myocardial infarction and stroke in patients with acute coronary syndromes (ACS). Yet, ticagrelor was found superior over clopidogrel in decreasing mortality in these patients and the underlying mechanisms are not entirely understood. Arterial thrombosis is the crucial step in ACS and the endothelium plays a pivotal role in mediating thrombus formation. However, possible off-target effects of $P2Y_{12}$ antagonists on the endothelium and its key procoagulant factors such as tissue factor (TF), have not yet been investigated. Frequently, patients with ACS have comorbidities such as atrial fibrillation (AF) requiring anticoagulant therapy to prevent embolism of thrombi originating from left atrial appendages (LAA). Similar to endothelial cells, activation of LAA endocardial cells induces procoagulant TF and plasminogen activator inhibitor-1 (PAI-1) expression favouring thrombus formation in patients with AF. Whether certain $P2Y_{12}$ antagonists possess antithrombotic properties on endocardial cells and may reduce thrombogenicity in AF patients has not yet been investigated.

2.2 Material and methods

Human aortic endothelial cells (HAECs) were incubated with ticagrelor or clopidogrel active metabolite (CAM) before stimulation with tumor necrosis factor-alpha (TNF-α); next, TF protein expression and enzyme activity as well as the underlying molecular mechanisms were investigated. Additionally, C57BL/6 mice were treated with ticagrelor or clopidogrel before endothelial TF expression was determined in common carotid arteries and photochemical-induced arterial thrombosis was compared between the groups. Finally, endocardial cells were isolated from LAA of 14 patients with AF undergoing elective cardiac

surgery. Endocardial cells were treated with ticagrelor or CAM and stimulated with TNF-α and TF as well as PAI-1 expressions and enzyme activities were analysed.

2.3 Results

Ticagrelor, unlike CAM, decreased TNF-α-induced TF activity and expression via proteasomal degradation in HAECs. These effects were mediated via the signalling molecules phosphoinositide 3-kinase and p70s6 kinase and independently of the $P2Y_{12}$ receptor and the equilibrative nucleoside transporter 1 (ENT1). Likewise, ticagrelor, but not clopidogrel, reduced endothelial TF expression in common carotid arteries of C57BL/6 mice and prolonged time to arterial thrombosis; meanwhile, platelet inhibition, plasma TF activity and systemic coagulation were comparable between the two groups. In LAA endocardial cells from AF patients, ticagrelor, unlike CAM, decreased TNF-α-induced TF and PAI-1 protein expressions and enzyme activities.

2.4 Conclusions

Ticagrelor, but not clopidogrel, displays local antithrombotic properties on the endothelium and, compared with clopigorgel, reduces arterial thrombosis. Likewise, ticagrelor, but not CAM, exhibits local antithrombotic properties in AF patients by reducing expressions and activities of TF and PAI-1 in LAA endocardial cells. The specific antithrombotic properties of ticagrelor may contribute to the reduced mortality in ACS patients observed in clinical trials and may prevent systemic thromboembolism in patients with AF.

3 Introduction

3.1 Epidemiology of cardiovascular disease

Cardiovascular (CV) disease (CVD), including coronary heart disease and stroke, is the leading cause of death in Europe (Fig. 1)[1], the United States of America[2] and worldwide.[3] In Europe, 4 million deaths per year are attributed to CVD, thereby representing 45 % of all deaths[1]. Interestingly, absolute and relative numbers of CV mortality are greater in women than in men, which is due to a higher number of cerebrovascular and “other CVD”, whereas coronary heart disease is comparable among both sexes (Fig. 1).[1]

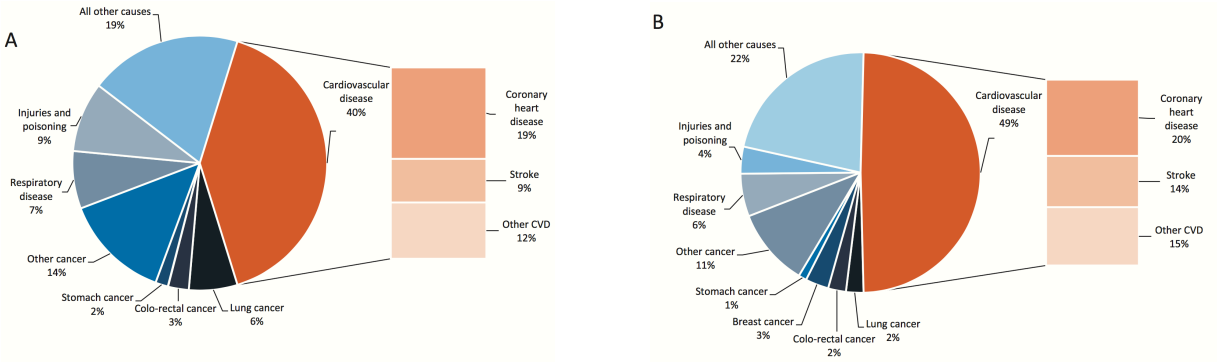

Figure 1. Deaths due to major causes in (A) men and (B) women in Europe. Source:[1]

From 1990 to 2013, CV mortality increased from 12.3 to 17.3 million deaths worldwide, representing 25.9 % and 31.5 % of all non-communicable diseases, respectively.[1] One explanation for this observation represents the ageing population[4] since age is a major risk factor for the development of CVD.[5] Ageing results from improved healthcare[6] and better lifestyle conditions[7] and is expected to continue in the near future causing substantial increase of disease burden and healthcare costs.[4] Besides coronary heart disease and stroke, atrial fibrillation represents the most common cardiac arrhythmia with a prevalence of 1.5 – 2 % in the general population and occurs at an average age of 75 – 85 years.[8] In addition to the increased risk for mortality, thromboembolic complication, such as stroke, cause a significant disease burden and healthcare costs, which are estimated to further increase in the next decades.[8]

3.2 Endothelial function and dysfunction

Vessel wall

The vascular wall consists of an inner layer of endothelial cells, followed by vascular smooth muscle cells (VSMC) and adventitial cells[9] (Fig. 2). The endothelial layer represents a roughly 0.2 μm thick monolayer covering the whole vasculature and thereby an area of approximately 3000 – 6000 m^2, whereas the majority of cells are considered microvascular endothelial cells covering particularly capillaries.[10] The volume of the entire endothelium is comparable to the volume of the liver.[11] The endothelium preserves blood fluidity by preventing thrombus formation, adjusts tissue perfusion by regulating vascular tone, regulates vascular permeability and inflammatory responses, and plays a pivotal role during angiogenesis.[11]

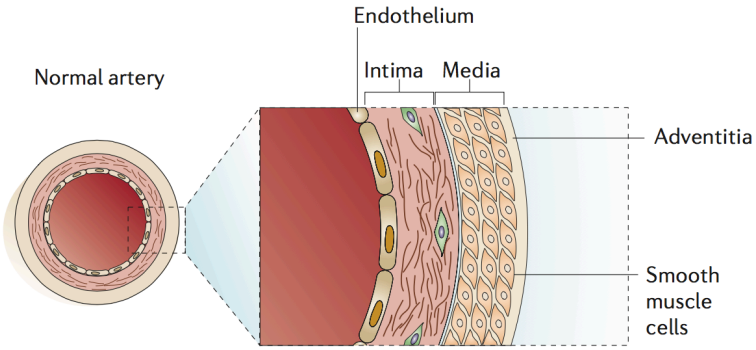

Figure 2. Arterial wall structure. The arterial wall is divided into the tunica intima containing ECs, the tunica media containing VSMCs and the tunica adventitia containing adventitial cells. EC = endothelial cell, VSMC = vascular smooth muscle cell. Source:[9]

Endothelial function

In healthy conditions, the endothelium prevents thrombus formation by physically separating platelets and coagulation factors from subendothelial prothrombotic mediators, such as collagen and tissue factor (TF)[12], and by expressing anticoagulant mediators such as nitric oxide (NO)[13,14], prostacyclin (PGI_2)[15], ectonucleotidase CD39[16], tissue factor pathway inhibitor (TFPI)[17] and antithrombin III[18] among others. During resting state, endothelial cells do not interact with leukocytes since expression of adhesion molecules is down regulated and

chemokines are not secreted.[19] Vascular tone is regulated via various vasoactive substances causing vasodilatation, such as NO, prostacyclin, adenosine and hydrogen peroxide (H_2O_2) or mediators causing vascular contraction, such as thromboxane A2, endothelin, angiotensin II, superoxide anion (O^2-) but also hydrogen peroxide.[11]

Endothelial dysfunction and reactive oxygen species

Endothelial dysfunction is characterised by reduced vasodilatatory properties or a shift toward a proinflammatory or a prothrombotic state[20]; it is associated with aging, diabetes, hypertension and atherosclerosis[11] and predicts CV outcome in patients with peripheral arterial disease[21], atherosclerosis[22], ACS[23] and heart failure.[24]

Despite the complexity of the pathogenic mechanisms leading to ED, oxidative stress is considered a central player in this respect.[11] Relevant reactive oxygen species (ROS) for vascular pathology include NO, superoxide anion, hydrogen peroxide and peroxynitrite ($ONOO^-$).[25] NO is produced by endothelial NO synthase[26] and by inducible NO synthase in endothelial cells, macrophages and VSMCs during inflammatory state.[27] It causes endothelial-dependent vasodilatation[13,14], inhibits platelet adhesion[28] and aggregation[29], and adhesion of leukocytes by inhibiting the expression of adhesion molecules.[20] Superoxide anion is produced by various enzymes including NO synthase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase[30] and occurs in all cell types. In the presence of superoxide anion, NO reacts to $ONOO^-$, which causes oxidation of proteins, deoxyribonucleic acids and lipids[31] leading to the formation of oxidized low-density lipoprotein (LDL)[25] among others. Alternatively, $O2^-$ is reduced to H_2O_2 by superoxide dismutase and finally dismutated to water and oxygen by catalase or glutathione peroxidase.[11] In the presence of copper and ion or superoxide however, H_2O_2 forms highly reactive hydroxyl radicals (OH).[11]

ROS affect endothelium-derived vasorelaxation by uncoupling NO synthase thus reducing the bioavailability of NO and by inhibiting the downstream NO target guanylyl cyclase.[11] Also, ROS cause vasoconstriction by multiple mechanisms including increased release of calcium from the sarcoplasmatic reticulum[32] and activation of cyclooxygenase-1 and subsequent production of endothelium-derived contracting factors.[33] Further, ROS induce the expression of endothelial adhesion molecules[34] and monocyte chemoattractant protein-1[35] enhancing leukocyte-endothelium interaction and thereby modulating inflammatory responses.

3.3 Atherosclerosis

Atherosclerosis may be defined as a thickening and loss of elasticity of arterial walls due to atherosclerotic plaque formation[36,37] typically affecting the subendothelial intima of large and medium-sized arteries.[38] Atherosclerotic plaques consist of connective tissue, such as collagen and proteoglycans, cholesterol and phospholipids as well as cellular components including macrophages/foam cells, T-lymphocytes and VSMC[36-38] (Fig. 3).

ED, a shift of the endothelium towards a prothrombotic and proinflammatory state with a reduced vasodilatory capacity, is driven by classical CV risk factors such as dyslipidaemia, diabetes and hypertension[11] and is considered a precursor of atherosclerosis.[39] ED causes increased permeability and leukocytes adhesion to the endothelium[40] (Fig 3 B). During the early phase of plaque formation, intimal thickening occurs due to accumulation of connective tissue, particularly proteoglycans, followed by lipid deposition[41] (Fig. 3C).

Lipoproteins containing apolipoprotein B, such as LDL and presumably lipoprotein(a), are of particular importance for plaque development. LDL accumulates in the subendothelial space through binding to extracellular connective tissue, i.e. proteoglycans.[42] Interaction of LDL with proteoglycans depends on LDL size and density, whereas small and dense particles possess higher binding capacities.[42] LDL retention increases susceptibility of multiple lipoprotein modifications such as self-aggregation, cleavage and oxidation, thereby transforming LDL

particles proatherogenic, finally, leading to the progression of atherosclerotic plaque formation.[42] Indeed, oxidized LDL has been demonstrated in human atherosclerotic plaques.[43] From a clinical perspective, plasma levels of LDL strongly correlate with the development of atherosclerosis.[44] Consistently, reduction of LDL has been shown to reduce the risk for CV events[45] strongly supporting a causal concept between LDL cholesterol and atherosclerosis development and progression.

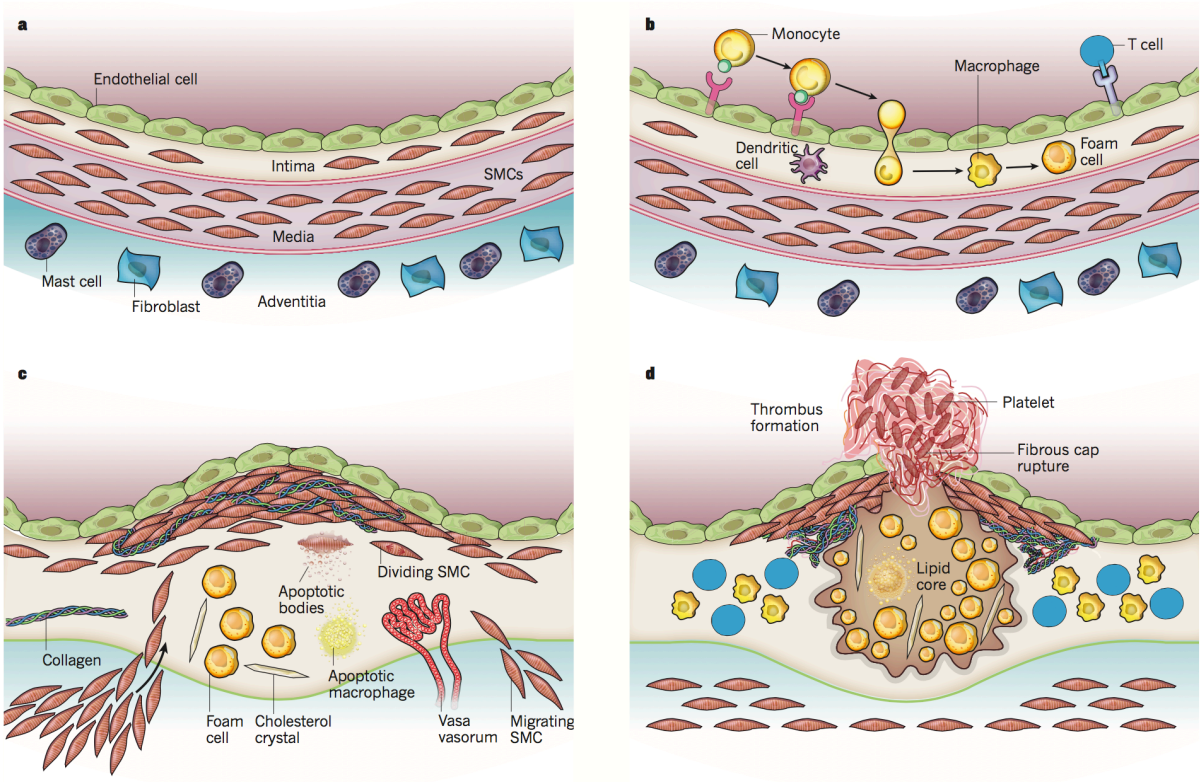

Figure 3. Atherosclerosis development. A) Healthy arterial wall. **B)** Initiation of atherosclerosis by transmigration of leukocytes and subsequent foam cell formation of monocytes through lipid uptake. **C)** Progression of atherosclerotic lesion through VSMC proliferation and migration, production of extracellular matrix connective tissue, apoptosis of macrophages and finally, accumulation of apoptotic bodies forming a necrotic core. **D)** Fibrous cap rupture and subsequent formation of an arterial thrombus through interaction of platelets and coagulation factors. Source: [36]

Endothelial transmigration of leukocytes including monocytes and T-lymphocytes (Fig. 3B) depends on the expression of endothelial adhesion molecules[40] and is mediated by chemoattractants[46,47], which can be stimulated by oxidized LDL.[46] Furthermore, both LDL

aggregation[48] and oxidation stimulate LDL uptake in macrophages[49] through scavenger receptors[50] finally leading to LDL degradation, intracellular lipid deposition and foam cell formation.[51] Scavenging of oxidized LDL through macrophages may reduces detrimental effects on surrounding cells, such as endothelial cells or VSMC; nevertheless, progressive lipid deposition magnifies inflammatory responses and overcomes such compensatory mechanisms of macrophages.[40] After adherence and migration, T-lymphocytes are in turn activated by macrophages through presentation of oxidized LDL[52] and secretion of cytokines including interferon-y and tumor necrosis factor-alpha (TNF-α).[53] Likewise, monocytes that transform to proinflammatory M2 macrophages secrete proinflammatory cytokines[54] thereby further enhancing the inflammatory response. Some foam cells undergo apoptosis followed by necrosis and release lipids, which together with necrotic cell components form the necrotic core in atherosclerotic plaques.[55]

In addition to monocytes and leukocytes, VSMC contribute to atherosclerotic plaque formation either by proliferation of VSMC in the intima, present in human arteries[56], or migration from the VSMC layer of the vessel[37] (Fig. 3C). Recently it has been proposed that in addition to local VSMC, bone marrow-derived stem cells contribute to atherosclerotic vascular pathology by homing, differentiation and migration.[57] VSMC secrete connective tissue like collagen and elastin, which together build the fibrous cap covering the atherosclerotic plaques and preventing plaque rupture[36] (Fig. 3D).

3.4 Tissue factor and initiation of the coagulation cascade

Tissue factor

TF is a transmembrane glycoprotein, which initiates the extrinsic coagulation cascade by binding coagulation factor VII[58], thereby activating factor IX and X[59] finally resulting in thrombin and subsequent fibrin formation.[60] Full length TF is a 47-kDa protein encoded by 6 exons located at chromosome 1 and comprises 263 amino acids, whereas the extracellular

domain consists of residues 1 – 219, the transmembrane domain of residues 220 – 242 and the cytoplasmic domain of residues 243 – 263.[58] The factor VII binding side is located at the extracellular domain.[61] In addition to full length TF, alternatively spliced TF has been described, which lacks exon 5.[62] Therefore, alternatively spliced tissue factor contains only 206 amino acids, whereas residues 1 – 166 are identical to full length TF[62] representing its extracellular domain and contain the factor VII binding side;[61] thus alternatively spliced TF is considered to be able to initiate coagulation.[62] The remaining residues 167 – 206 differ from full length TF due to a frameshift mutation and represent a unique C-terminus.[62] Since the transmembrane domain is not expressed, alternatively spliced TF is soluble and circulates in blood.[62] Possible sources of alternatively spliced TF include monocytes[62] and endothelial cells.[63] Alternatively spliced TF has been detected in human thrombi and is believed to contribute to thrombogenicity;[62] however, its procoagulant activity was not proven by others[64,65] and therefore its role in thrombosis is unclear. In addition to active cell surface TF, inactive and so called “encrypted” TF has been described.[66] Like active TF, it binds factor VII, however, does not activate factor X sufficiently to prompt procoagulant activity.[66] Transformation to the active form is mediated by protein-disulfide isomerase[67] and has been shown to be crucial for fibrin generation and thrombus formation in mice.[68]

TF is expressed in endothelial cells[69], VSMCs, the adventitia[70], monocytes[71], platelets and on circulating TF-containing microparticles released from platelets[72,73], endothelial cells and monocytes.[74] Furthermore it is expressed not only in the necrotic core of atherosclerotic plaques, but also in macrophages and the endothelium covering human atherosclerotic plaques.[70,75,76] In physiological conditions only spare amount of TF are found in endothelial cells; however, during pathological conditions, such as high shear flow as occurring in a stenosed vessel[77] and in the presence of various inflammatory cytokines such TNF-α and interleukin 1[78] as well as thrombin[79], CD40 ligand[80] and oxidized LDL[81], TF expression is

increased in endothelial cells. Likewise, interleukin 1 induces tissue factor expression in monocytes[82] and CD40 ligand in monocytes[83] and VSMCs.[84]

Initiation of the coagulation cascade

Tissue factor represents a cellular receptor for the plasma serine protease factor VII or the activated form, factor VII_a[85] (Fig. 4). Binding of TF enhances factor VII activation to VII_a[86] and binding of TF is essential to increases factor VII_a enzyme activity in order to activate factor IX and factor X to factor IX_a and X_a, respectively.[85] Activation of factor VII to factor VII_a is further mediated by factor IX_a and X_a[87] as well as factor VII_a itself.[88] Next, factor X_a forms the prothrombinase complex with factor V_a on TF-containing cells,[89] thereby transforming small amounts of prothrombin to thrombin, which subsequently amplifies the coagulation signal by activating factor V, VIII and XI on platelet surfaces[90] (Fig. 4). Finally, thrombin transforms fibrinogen to fibrin[91] and activates platelets by cleavage of protease-activated receptors.[92]

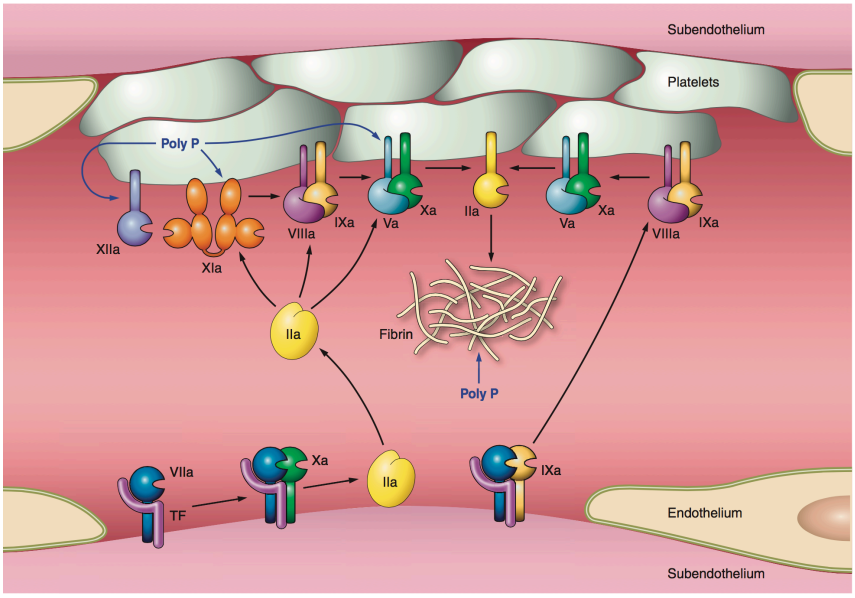

Figure 4. Coagulation system. TF binds activated factor VII (VII_a) and activates factor X and factor IX. Factor X_a leads to the production of small amounts of thrombin, which in turn activates factor V (cofactor of factor X), factor VIII (cofactor of factor IX) and factor XI. Factor XI_a further enhances factor IX activation. Factor IX_a together with cofactor $VIII_a$ (tenase complex) increase factor X activation. Finally, factor X_a binds factor V_a (prothrombinase complex) and increases thrombin production substantially. TF = tissue factor. Source:[86]

In order to preserve an antithrombotic endothelial cell surface in physiologic condition, TFPI counteracts TF-mediated activation of coagulation and subsequent thrombus formation[93]. TFPI is expressed in endothelial cells under quiescent conditions and, like TF, can be upregulated upon activation by inflammatory cytokines.[94] TFPI directly inhibits factor X_a,[93] most efficiently if factor X_a is integrated in the prothrombinase complex with factor V_a, calcium ions and phospholipids.[95] In addition, TFPI inhibits the TF/factor VII($_a$) through complexing with TF/factor VII($_a$) and X_a.[93]

Experimental and clinical studies

Experimental data revealed reduced arterial thrombus formation in low-TF expressing mice thus suggesting a pivotal role of vascular TF in thrombus formation.[96] Transgenic mice deficient in murine TF and expressing 1% of human tissue factor, revealed prolonged arterial occlusion times after photochemical injury of the carotid artery using bengal rose.[96] Neither did transplantation of low-TF bone marrow to wild-type animals increase, nor did transplantation of wild-type bone marrow to low-TF animals decrease arterial occlusion times confirming the relevance of vascular- rather than blood cell-derived TF in arterial thrombus formation.[96] In addition, anti-TF antibody treated rabbits displayed reduced arterial thrombus formation after injury and subsequent stenosis of the common carotid artery.[97] In clinical studies, increased levels of TF have not only been associated with cardiovascular risk factors including smoking[98], hypertension[99], diabetes[100] and dyslipidaemia[101] but also with acute coronary syndrome (ACS)[102] emphasizing the clinical relevance of TF.

3.5 Platelets and the $P2Y_{12}$ receptor

Platelets

Human platelets are anucleated blood cells derived from bone marrow magakaryocytes with an average life span of 9 to 10 days.[103] Despite the lack of DNA, platelets contain

megakaryocyte-derived messenger RNA[104] and are capable of protein synthesis.[105] One-hundred-fifty x 10^9 to 400 x 10^9 human platelets per litre of blood[103] are present in the circulation, which are in turn surrounded by 3000 – 6000 m^2 of endothelial surface.[10] In physiological conditions, the endothelium prevents platelet adhesion and aggregation by separating platelets from the subendothelial matrix, by inhibiting platelets through NO[13,14] and PGI_2[15] and by inhibiting platelet agonists through antithrombin III[18] and adenosine diphosphate (ADP) metabolism, among others.[106] During resting state platelets appear round/oval whereas upon endothelial damage, platelets adhere to the side of injury, become activated and aggregate, which results in profound shape changes such as cellular spreading.[12] Platelet are crucial to sustain haemostasis in case of injury; however, they may cause thrombus formation, subsequent vascular occlusion and tissue ischemia in pathological conditions.[12]

In case of vascular injury or endothelial disruption, subendothelial extracellular matrix containing different types of collagens, von Willebrand factor (vWF), fibronectin and laminin is exposed to the blood stream[107] (Fig. 5A). In high shear conditions as observed in the arterial circulation or in stenosed vessels, binding of the platelet receptor glycoprotein (GP) Ibα, part of the GPIb-V-IX complex, to extracellular vWF or plasma-derived vWF bound to subendothelial collagen, is crucial for platelet tethering.[108] Yet, additional interactions of platelet GPVI and/or integrin $\alpha_2\beta_1$ with collagen, platelet integrin $\alpha_5\beta_1$ with fibronectin or platelet integrin $\alpha_6\beta_1$ with laminin is required for platelet adhesion.[109] Adhesion of platelets to extracellular matrix proteins, particularly collagen and vWF, activates the platelet integrin $\alpha_{IIb}\beta_3$ receptor allowing binding of several ligands such as soluble vWF, fibrinogen and fibrin and by that cross-linkage of platelets, a key step in firm adhesion and platelet aggregation[12,107] (Fig. 5B). In parallel platelets produce thromboxane A2 and release dense granules containing ADP and α granules containing vWF, fibrinogen, fibronectin, p-selectin and interleukin 1 beta (Fig. 5A), which amplify platelet activation (ADP, thromboxane),

facilitate platelet-endothelium interaction (vWF, fibronectin), platelet-leukocyte interaction (fibronectin, fibrinogen p-selectin), leukocyte-endothelium interaction (p-selectin, interleukin 1 beta), platelet aggregation (vWF, fibrinogen) and finally, fibrin clot formation (fibrinogen, factor V).[12,109]

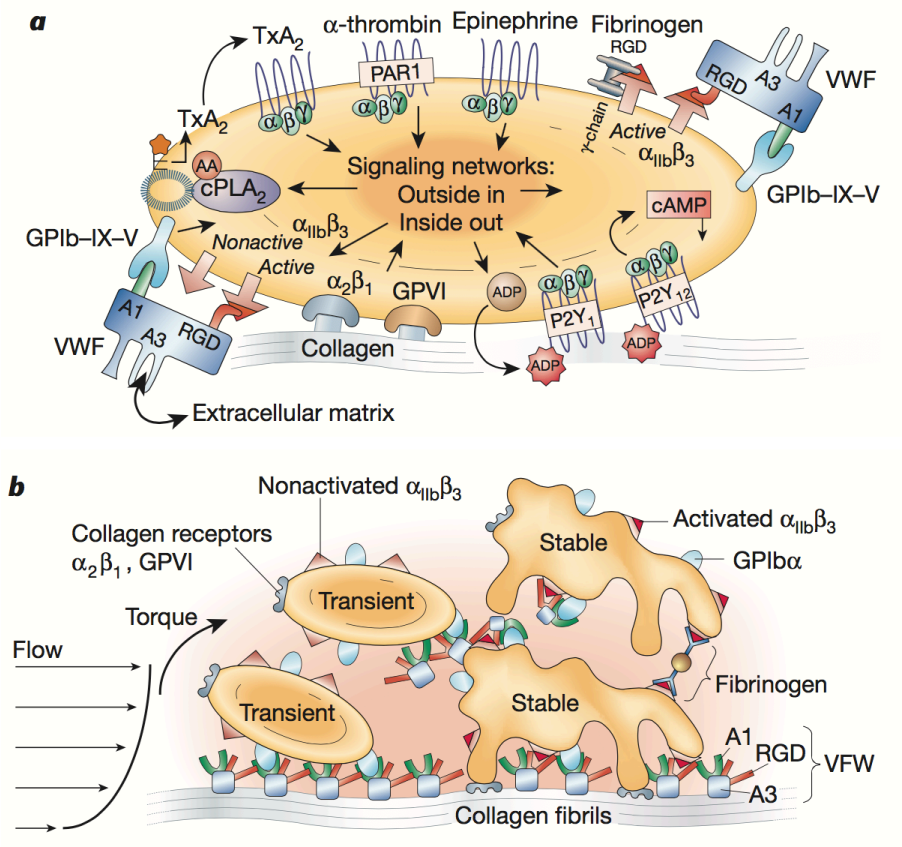

Figure 5. Platelet activation and aggregation. A) Platelet tethering to the vessel wall is mediated via GPIb-V-IX binding to subendothelial vWF. In addition, platelet GPVI and/or integrin $\alpha_2\beta_1$ binding to collagen is required for platelet adhesion and leads to activation of the $\alpha_{IIb}\beta_3$ receptor, thromboxane A2 production and release of dense and α granules containing ADP, vWF, fibrinogen, fibronectin, p-selectin and interleukin 1 beta further enhancing platelet activation and aggregation. In addition, platelets are activated by other agonists such as thrombin. B) Finally, activated $\alpha_{IIb}\beta_3$ receptor binds soluble vWF, fibrinogen and fibrin and mediates cross-linkage and thus, platelet aggregation. ADP = adenosine diphosphate, GP = glycoprotein, vWF = von Willebrand factor. Source:[12]

Adenosine diphosphate and the P2Y receptors

The released of ADP from dense granules, stimulated by collagen and thrombin,[110] causes significant amplification of platelet activation by auto- or paracrine binding to platelet purine

receptors $P2Y_1$[111] and $P2Y_{12}$.[112] Purine and pyrimidine receptors are divided in P1 receptors selective for adenosine and P2 receptors, which are further divided in the ligand-gated ion channels P2X receptors and the G protein-coupled P2Y receptors.[113] Binding of ADP to the metabotropic G_q-coupled $P2Y_1$ receptor results in phospholipase C activation, subsequent increase of inositol triphosphate and rise of cytosolic calcium from intracellular stores.[113] Instead, activation of the metabotropic G_i-coupled $P2Y_{12}$ receptor leads to an inhibition of the adenylyl cyclase and subsequent decrease of cyclic adenosine monophosphate.[113] In addition, the $P2Y_{12}$ receptor activates phosphoinositide 3-kinase (PI3K) and thereby the $\alpha_{IIb}ß_3$ receptor.[114] Activation of the $P2Y_1$ leads to platelet shape change and transient aggregation, whereas activation of the $P2Y_{12}$ receptor results in dense granule secretion and sustained platelet aggregation.[110] In summary, activation of both receptors is necessary to grant full platelet aggregation since blockade of each receptor individually reduces platelet aggregation.[115] Interestingly, inhibition of both receptors in parallel shows synergistic effects.[116]

3.6 $P2Y_{12}$ receptor antagonists

Due to the crucial role of platelets in arterial thrombus formation, platelet antagonists are routinely used in the clinic to reduce thrombogenesis and prevent cardiovascular complications such as myocardial infarction (MI) and stroke.[6] Beside from established platelet antagonists including the cyclooxygenase-1 antagonist aspirin[6], integrin $\alpha_{IIb}ß_3$ antagonists[117] and emerging inhibitors of GPIV[118], GPIb[119] and thromboxane A2 receptors[120], platelet ADP receptor $P2Y_{12}$ antagonists have been shown to reduce major adverse cardiac events in large clinical trials.[121-124] Currently four $P2Y_{12}$ receptor antagonists are approved for clinical use including clopidogrel, prasugrel, ticagrelor and cangrelor, whereas the first approved $P2Y_{12}$ receptor antagonist ticlopidine has been replaced due to adverse haematological side effects[125] and development of elinogrel was discontinued recently.[126] $P2Y_{12}$ receptor antagonists can be divided into thienopyridines including ticlopidine,

clopidogrel and prasugrel, in cyclopentyl-triazolo-pyrimidines including ticagrelor and in the adenosine triphosphate analogue cangrelor.[127]

The thienopyridines clopidogrel and prasugrel

Thienopyridines are indirect pro-drugs requiring conversion to the active metabolite by hepatic cytochrome P450 enzymes before irreversibly binding the $P2Y_{12}$ receptor.[127] Pharmacokinetic and pharmacodynamic properties differ significantly among $P2Y_{12}$ receptor antagonist. Clopidogrel not only exhibits weak inhibition of ADP-induced platelet aggregation with great inter-individual variability[128] but also 15 to 30 % of patients respond only marginally to clopidogrel treatment.[129] Importantly, higher platelet reactivity to ADP despite clopidogrel-treatment is associated with an increased risk of major adverse cardiac events.[129] Such non-responsiveness might be due to incompliance, differences in intestinal absorption and $P2Y_{12}$ receptor polymorphisms;[129] however, it has been proposed that variations in the cytochrome P450 3A4 considerably influences hepatic activation of clopidogrel.[130] Surprisingly, treatment adjustment in non-responders to clopidogrel did not improve cardiovascular outcome.[131]

Compared to clopidogrel, prasugrel shows a faster onset and higher level of platelet inhibition as well as a reduced inter-individual variability and lower non-responsiveness[132,133] due to a more efficient conversion of prasugrel to its active metabolite.[134] Consequently, prasugrel, compared with clopidogrel, reduced the primary composite endpoint, the rate of death from cardiovascular causes, non-fatal MI, or non-fatal stroke in ACS patients.[122] On the other hand, prasugrel increased major bleeding events and therefore, did not reduce overall mortality in these patients.[122]

The cyclopentyl-triazolo-pyrimidine ticagrelor

Ticagrelor and cangrelor are direct acting drugs, do not need hepatic transformation and bind to the $P2Y_{12}$ receptor reversibly.[127] Ticagrelor, similarly to prasugrel, exhibits a faster onset, a greater level of and a more rapid offset of inhibition of ADP-induced platelet aggregation.[135]

Due to reversible binding, ticagrelor may be more protective from platelet aggregation in patients with high platelet turnover due to a more sustained platelet inhibition of newly formed (reticulated) platelets.[136] Patients with higher number of reticulated platelets are at higher risk for cardiovascular events.[137] Correspondingly, aggregation of immature platelets correlated with prasugrel, but not ticagrelor-treated patients with ACS.[138] In large randomized clinical trials, ticagrelor proved higher efficacy, compared with clopidogrel in ACS patients; ticagrelor reduced the primary composite endpoint, death from vascular causes, MI and stroke without increasing the primary safety endpoint, major bleedings although fatal intracranial and non-intracranial bleeds were higher in ticagrelor-treated patients[121]. Importantly, ticagrelor, compared with clopidogrel, also reduced overall mortality.[121] Furthermore, in patients with prior MI, ticagrelor, compared with clopidogrel, reduced cardiovascular death, MI and stroke, but also increased major bleedings.[139]

Pleiotropic effects of $P2Y_{12}$ receptor antagonists

Thienopyridines were shown to have multiple pleiotropic effects.[140] In apolipoprotein E-deficient mice prone to atherosclerosis, clopidogrel treatment reduced atherosclerotic lesion size by roughly one third, compared to control animals.[141] Anti-inflammatory properties have been described for prasugrel; in a mouse model for endotoxic shock syndrome, prasugrel reduced platelet p-selectin expression and platelet-leukocyte interaction as well thromboxane 2 and TNF-α production.[142] Both, ticlopidine and clopidogrel were found to mediate endothelial-dependent vasodilatation in pre-contracted arteries of rats.[143] In patients with coronary artery disease and peripheral endothelial dysfunction, single dosages of clopidogrel dose-dependently reduced endothelial dysfunction as assessed by flow-mediated dilatation of the brachial artery.[144]

Like for thienopyridines, pleiotropic effects have been described also for ticagrelor.[140,145] In addition to the $P2Y_{12}$ receptor, ticagrelor, but not thienopyridines or cangrelor, binds to the equilibrative nucleoside transporter 1 (ENT1),[146] which is expressed in red blood cells among

many others,[147] and reduces adenosine uptake.[146] Consequently, ticagrelor decreased adenosine uptake in human red blood cells[148] and increased plasma levels of adenosine in ACS patients treated with ticagrelor.[149] Adenosine inhibits platelet activation[150] and was found to contribute to platelet inhibition in human whole blood treated with ticagrelor.[151] Furthermore, ticagrelor, but not prasugrel or clopidogrel, reduced VSMC contraction in rats;[152] Treatment with ticagrelor revealed anti-inflammatory properties by reducing pulmonary oedema, neutrophil recruitment and lung damage in mice exposed to experimental abdominal sepsis.[153] In addition, ticagrelor, unlike clopidogrel, reduced MI in rats[154] and in pigs in an adenosine-dependant manner.[155] In patients with prior ACS, ticagrelor, compared with clopidogrel and prasugrel, improved peripheral arterial function after forearm ischemia.[156] Such additional pleiotropic effects may in part contribute to the differences in outcomes observed in randomized controlled trials comparing $P2Y_{12}$ inhibitors. [121,139]

3.7 Atherothrombosis

Arterial thrombosis on top of an atherosclerotic lesion is the key event in ACS and results from interactions between the vessel wall, blood cells including platelets, red blood cells and leukocytes and the coagulation system.[86,157-160]

Atherosclerotic plaques

Complex atherosclerotic lesions prone to rupture and to cause arterial thrombosis usually present a thin fibrous caps, are large in size and cause a small luminal area;[161] they typically cause expansive vascular remodelling and exhibit low calcification.[162] Furthermore, they contain a large lipid core, numerous leukocytes and fewer VSMC.[163]

During the early stage of plaque development, atherosclerosis causes vascular enlargement by expansive growth, which compensates vascular stenosis through the newly formed plaque; therefore, stenosis occurs late during plaque development.[164] Plaque rupture rather

than superficial erosions of the fibrous caps overlying the lipid or so-called necrotic core, causes the majority of fatal coronary thrombosis.[165]

Plaque rupture is associated with thin fibrous caps[166] and its stability is believed to be mediated primarily through collagen, which is synthetized by VSMCs and degraded by some matrix-metalloproteinases.[159] Accumulation of T-lymphocytes and macrophages, however, reduces collagen synthesis by inhibiting VSMCs and increase collagen breakdown by producing matrix-metalloproteinases, respectively.[159] In contrast to plaque rupture, plaque erosion is believed to be triggered primarily by endothelial apoptosis due to increased oxidative stress from activated leukocytes, which also increases up-regulation of pro-coagulant TF.[159] Interestingly, statin therapy not only reduces plasma low-density lipoprotein and lipid content of atherosclerotic plaques but also decreases atherosclerotic plaque macrophage activity, which may contribute to its well-defined cardiovascular protective effects.[167] Also, anti-inflammatory therapy with low-dose colchicine reduced cardiovascular events in patients with stable coronary artery disease[168] highlighting the importance of inflammation in atherosclerosis and subsequent arterial thrombosis.

Platelet adhesion, activation and recruitment

Upon plaque erosion or rupture due to underlying inflammatory mechanisms[159], subendothelial extracellular matrix is exposed. In high shear conditions, as occurring in the arterial vasculature, vWF is essential for platelet tethering[108] and subsequent binding of various platelet receptors to subendothelial collagen, fibrinogen and laminin enables platelet adhesion[109]. Platelet adhesion triggers release of platelet granules containing ADP and thromboxane, which enhances platelet activation.[12,109] Next, integrin $\alpha_{IIb}\beta_3$ receptors become activated and, together with platelet GPIb, binds vWF and other soluble molecules, whereby additional platelets are recruited and aggregate at the side of plaque erosion or rupture.[12,107]

Initiation, amplification and propagation of the coagulation cascade

In parallel to platelet recruitment, the coagulation cascade is initiated by TF.[157] Exposure of TF and subsequent binding of factor VII/factor VII_a[85] activates factor IX and factor X.[85] Next, factor X_a forms the prothrombinase complex with factor V_a on TF-containing containing cells[89], which leads to the formation of small amounts of thrombin.[91]

Following initiation of the coagulation cascade, thrombin not only leads to platelet activation via cleavage of protease-activated receptors 1 and 4[92] but also activates co-factor V, co-factor VIII and factor XI on platelet surfaces thereby amplifying the procoagulant signal.[90] During the propagation phase, large amounts of thrombin are produced.[90] After thrombin-mediated activation of factor XI to XI_a, factor XI_a activates factor IX to IX_a, which binds factor $VIII_a$ and thus forms the tenase complex activating factor X to X_a; factor X_a complexes with V_a and increases thrombin.[86] Finally, thrombin transforms fibrinogen to fibrin, which subsequently polymerizes to fibrin strands.[91] Lastly, after activation by thrombin among others, factor $XIII_a$ stabilizes polymerized fibrin strands to form a stable platelet clot.[169]

3.8 State of research in the field

In addition to aspirin, $P2Y_{12}$ receptor antagonists including clopidogrel, prasugrel and ticagrelor are crucial to reduce MI, stroke and CV death in patients suffering an ACS as shown in large randomized controlled trials.[121-123] Both prasugrel and ticagrelor exhibit greater inhibition of ADP-induced platelet aggregation, as compared with clopidogrel.[132,135] Yet, despite comparable efficacy in platelet inhibition[170] only ticagrelor, but not prasugrel, reduced overall mortality in ACS patients, as compared with clopidogrel.[121,122] Recently described pleiotropic anti-inflammatory[153] and vasodilative effects[152] of ticagrelor as well as the ability to inhibit the adenosine transporter ENT1, may in part explain this observation. Nevertheless, $P2Y_{12}$ receptors are not only expressed in platelets but also in the vessel

wall[171] and platelet-independent vascular effects of $P2Y_{12}$ antagonists on arterial thrombogenesis remain unknown.

4 References

1. Townsend N, Wilson L, Bhatnagar P, Wickramasinghe K, Rayner M, Nichols M. Cardiovascular disease in Europe: epidemiological update 2016. European heart journal 2016;37:3232-45.

2. Mozaffarian D, Benjamin EJ, Go AS, et al. Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. Circulation 2016;133:e38-360.

3. Lozano R, Naghavi M, Foreman K, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 2012;380:2095-128.

4. Heidenreich PA, Trogdon JG, Khavjou OA, et al. Forecasting the future of cardiovascular disease in the United States: a policy statement from the American Heart Association. Circulation 2011;123:933-44.

5. Sniderman AD, Furberg CD. Age as a modifiable risk factor for cardiovascular disease. Lancet 2008;371:1547-9.

6. Windecker S, Kolh P, Alfonso F, et al. 2014 ESC/EACTS Guidelines on myocardial revascularization: The Task Force on Myocardial Revascularization of the European Society of Cardiology (ESC) and the European Association for Cardio-Thoracic Surgery (EACTS)Developed with the special contribution of the European Association of Percutaneous Cardiovascular Interventions (EAPCI). European heart journal 2014;35:2541-619.

7. Perk J, De Backer G, Gohlke H, et al. European Guidelines on cardiovascular disease prevention in clinical practice (version 2012). The Fifth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of nine societies and by invited experts). European heart journal 2012;33:1635-701.

8. Camm AJ, Lip GY, De Caterina R, et al. 2012 focused update of the ESC Guidelines for the management of atrial fibrillation: an update of the 2010 ESC Guidelines for the

management of atrial fibrillation. Developed with the special contribution of the European Heart Rhythm Association. European heart journal 2012;33:2719-47.

9. Watkins H, Farrall M. Genetic susceptibility to coronary artery disease: from promise to progress. Nature reviews Genetics 2006;7:163-73.

10. van Hinsbergh VW. Endothelium--role in regulation of coagulation and inflammation. Seminars in immunopathology 2012;34:93-106.

11. Feletou M, Vanhoutte PM. Endothelial dysfunction: a multifaceted disorder (The Wiggers Award Lecture). American journal of physiology Heart and circulatory physiology 2006;291:H985-1002.

12. Ruggeri ZM. Platelets in atherothrombosis. Nature medicine 2002;8:1227-34.

13. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proceedings of the National Academy of Sciences of the United States of America 1987;84:9265-9.

14. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 1987;327:524-6.

15. Cheng Y, Austin SC, Rocca B, et al. Role of prostacyclin in the cardiovascular response to thromboxane A2. Science 2002;296:539-41.

16. Marcus AJ, Broekman MJ, Drosopoulos JH, et al. The endothelial cell ecto-ADPase responsible for inhibition of platelet function is CD39. The Journal of clinical investigation 1997;99:1351-60.

17. Osterud B, Bajaj MS, Bajaj SP. Sites of tissue factor pathway inhibitor (TFPI) and tissue factor expression under physiologic and pathologic conditions. On behalf of the Subcommittee on Tissue factor Pathway Inhibitor (TFPI) of the Scientific and Standardization Committee of the ISTH. Thrombosis and haemostasis 1995;73:873-5.

18. Bauer KA, Rosenberg RD. Role of antithrombin III as a regulator of in vivo coagulation. Seminars in hematology 1991;28:10-8.

19. Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. Nature reviews Immunology 2007;7:803-15.

20. Vanhoutte PM, Shimokawa H, Tang EH, Feletou M. Endothelial dysfunction and vascular disease. Acta Physiol (Oxf) 2009;196:193-222.

21. Gokce N, Keaney JF, Jr., Hunter LM, et al. Predictive value of noninvasively determined endothelial dysfunction for long-term cardiovascular events in patients with peripheral vascular disease. Journal of the American College of Cardiology 2003;41:1769-75.

22. Chan SY, Mancini GB, Kuramoto L, Schulzer M, Frohlich J, Ignaszewski A. The prognostic importance of endothelial dysfunction and carotid atheroma burden in patients with coronary artery disease. Journal of the American College of Cardiology 2003;42:1037-43.

23. Fichtlscherer S, Breuer S, Zeiher AM. Prognostic value of systemic endothelial dysfunction in patients with acute coronary syndromes: further evidence for the existence of the "vulnerable" patient. Circulation 2004;110:1926-32.

24. Heitzer T, Baldus S, von Kodolitsch Y, Rudolph V, Meinertz T. Systemic endothelial dysfunction as an early predictor of adverse outcome in heart failure. Arteriosclerosis, thrombosis, and vascular biology 2005;25:1174-9.

25. Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS. Circulation 2003;108:1912-6.

26. Bredt DS, Snyder SH. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proceedings of the National Academy of Sciences of the United States of America 1990;87:682-5.

27. Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. Circulation 2004;109:III27-32.

28. Radomski MW, Palmer RM, Moncada S. The role of nitric oxide and cGMP in platelet adhesion to vascular endothelium. Biochemical and biophysical research communications 1987;148:1482-9.

29. Furlong B, Henderson AH, Lewis MJ, Smith JA. Endothelium-derived relaxing factor inhibits in vitro platelet aggregation. British journal of pharmacology 1987;90:687-92.

30. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circulation research 2000;87:840-4.

31. Munzel T, Daiber A, Ullrich V, Mulsch A. Vascular consequences of endothelial nitric oxide synthase uncoupling for the activity and expression of the soluble guanylyl cyclase and the cGMP-dependent protein kinase. Arteriosclerosis, thrombosis, and vascular biology 2005;25:1551-7.

32. Suzuki YJ, Ford GD. Superoxide stimulates IP3-induced Ca2+ release from vascular smooth muscle sarcoplasmic reticulum. The American journal of physiology 1992;262:H114-6.

33. Vanhoutte PM, Boulanger CM. Endothelium-dependent responses in hypertension. Hypertension research : official journal of the Japanese Society of Hypertension 1995;18:87-98.

34. Weber C, Erl W, Pietsch A, Strobel M, Ziegler-Heitbrock HW, Weber PC. Antioxidants inhibit monocyte adhesion by suppressing nuclear factor-kappa B mobilization and induction of vascular cell adhesion molecule-1 in endothelial cells stimulated to generate radicals. Arteriosclerosis and thrombosis : a journal of vascular biology / American Heart Association 1994;14:1665-73.

35. Kunsch C, Medford RM. Oxidative stress as a regulator of gene expression in the vasculature. Circulation research 1999;85:753-66.

36. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature 2011;473:317-25.

37. Rader DJ, Daugherty A. Translating molecular discoveries into new therapies for atherosclerosis. Nature 2008;451:904-13.

38. Fuster V, Moreno PR, Fayad ZA, Corti R, Badimon JJ. Atherothrombosis and high-risk plaque: part I: evolving concepts. Journal of the American College of Cardiology 2005;46:937-54.

39. Vanhoutte PM. Endothelial dysfunction: the first step toward coronary arteriosclerosis. Circulation journal : official journal of the Japanese Circulation Society 2009;73:595-601.

40. Ross R. Atherosclerosis--an inflammatory disease. The New England journal of medicine 1999;340:115-26.

41. Nakashima Y, Fujii H, Sumiyoshi S, Wight TN, Sueishi K. Early human atherosclerosis: accumulation of lipid and proteoglycans in intimal thickenings followed by macrophage infiltration. Arteriosclerosis, thrombosis, and vascular biology 2007;27:1159-65.

42. Camejo G, Hurt-Camejo E, Wiklund O, Bondjers G. Association of apo B lipoproteins with arterial proteoglycans: pathological significance and molecular basis. Atherosclerosis 1998;139:205-22.

43. Yla-Herttuala S, Palinski W, Rosenfeld ME, et al. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. The Journal of clinical investigation 1989;84:1086-95.

44. Goldstein JL, Brown MS. The low-density lipoprotein pathway and its relation to atherosclerosis. Annual review of biochemistry 1977;46:897-930.

45. Cannon CP, Braunwald E, McCabe CH, et al. Intensive versus moderate lipid lowering with statins after acute coronary syndromes. The New England journal of medicine 2004;350:1495-504.

46. Rajavashisth TB, Andalibi A, Territo MC, et al. Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. Nature 1990;344:254-7.

47. Boring L, Gosling J, Chensue SW, et al. Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. The Journal of clinical investigation 1997;100:2552-61.

48. Khoo JC, Miller E, McLoughlin P, Steinberg D. Enhanced macrophage uptake of low density lipoprotein after self-aggregation. Arteriosclerosis 1988;8:348-58.

49. Stocker R, Keaney JF, Jr. Role of oxidative modifications in atherosclerosis. Physiological reviews 2004;84:1381-478.

50. Han J, Hajjar DP, Febbraio M, Nicholson AC. Native and modified low density lipoproteins increase the functional expression of the macrophage class B scavenger receptor, CD36. The Journal of biological chemistry 1997;272:21654-9.

51. Yu XH, Fu YC, Zhang DW, Yin K, Tang CK. Foam cells in atherosclerosis. Clinica chimica acta; international journal of clinical chemistry 2013;424:245-52.

52. Stemme S, Faber B, Holm J, Wiklund O, Witztum JL, Hansson GK. T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein. Proceedings of the National Academy of Sciences of the United States of America 1995;92:3893-7.

53. Hansson GK, Jonasson L, Seifert PS, Stemme S. Immune mechanisms in atherosclerosis. Arteriosclerosis 1989;9:567-78.

54. Bouhlel MA, Derudas B, Rigamonti E, et al. PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. Cell metabolism 2007;6:137-43.

55. Tabas I. Macrophage death and defective inflammation resolution in atherosclerosis. Nature reviews Immunology 2010;10:36-46.

56. Ikari Y, McManus BM, Kenyon J, Schwartz SM. Neonatal intima formation in the human coronary artery. Arteriosclerosis, thrombosis, and vascular biology 1999;19:2036-40.

57. Sata M, Saiura A, Kunisato A, et al. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. Nature medicine 2002;8:403-9.

58. Spicer EK, Horton R, Bloem L, et al. Isolation of cDNA clones coding for human tissue factor: primary structure of the protein and cDNA. Proceedings of the National Academy of Sciences of the United States of America 1987;84:5148-52.

59. Osterud B, Rapaport SI. Activation of factor IX by the reaction product of tissue factor and factor VII: additional pathway for initiating blood coagulation. Proceedings of the National Academy of Sciences of the United States of America 1977;74:5260-4.

60. Ruf W, Edgington TS. Structural biology of tissue factor, the initiator of thrombogenesis in vivo. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 1994;8:385-90.

61. Kelley RF, Costas KE, O'Connell MP, Lazarus RA. Analysis of the factor VIIa binding site on human tissue factor: effects of tissue factor mutations on the kinetics and thermodynamics of binding. Biochemistry 1995;34:10383-92.

62. Bogdanov VY, Balasubramanian V, Hathcock J, Vele O, Lieb M, Nemerson Y. Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein. Nature medicine 2003;9:458-62.

63. Szotowski B, Goldin-Lang P, Antoniak S, et al. Alterations in myocardial tissue factor expression and cellular localization in dilated cardiomyopathy. Journal of the American College of Cardiology 2005;45:1081-9.

64. Boing AN, Hau CM, Sturk A, Nieuwland R. Human alternatively spliced tissue factor is not secreted and does not trigger coagulation. Journal of thrombosis and haemostasis : JTH 2009;7:1423-6.

65. Censarek P, Bobbe A, Grandoch M, Schror K, Weber AA. Alternatively spliced human tissue factor (asHTF) is not pro-coagulant. Thrombosis and haemostasis 2007;97:11-4.

66. Le DT, Rapaport SI, Rao LV. Relations between factor VIIa binding and expression of factor VIIa/tissue factor catalytic activity on cell surfaces. The Journal of biological chemistry 1992;267:15447-54.

67. Versteeg HH, Ruf W. Tissue factor coagulant function is enhanced by protein-disulfide isomerase independent of oxidoreductase activity. The Journal of biological chemistry 2007;282:25416-24.

68. Cho J, Furie BC, Coughlin SR, Furie B. A critical role for extracellular protein disulfide isomerase during thrombus formation in mice. The Journal of clinical investigation 2008;118:1123-31.

69. Zeldis SM, Nemerson Y, Pitlick FA, Lentz TL. Tissue factor (thromboplastin): localization to plasma membranes by peroxidase-conjugated antibodies. Science 1972;175:766-8.

70. Wilcox JN, Smith KM, Schwartz SM, Gordon D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. Proceedings of the National Academy of Sciences of the United States of America 1989;86:2839-43.

71. Edwards RL, Rickles FR, Bobrove AM. Mononuclear cell tissue factor: cell of origin and requirements for activation. Blood 1979;54:359-70.

72. Muller I, Klocke A, Alex M, et al. Intravascular tissue factor initiates coagulation via circulating microvesicles and platelets. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2003;17:476-8.

73. Siddiqui FA, Desai H, Amirkhosravi A, Amaya M, Francis JL. The presence and release of tissue factor from human platelets. Platelets 2002;13:247-53.

74. Shet AS, Aras O, Gupta K, et al. Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes. Blood 2003;102:2678-83.

75. Thiruvikraman SV, Guha A, Roboz J, Taubman MB, Nemerson Y, Fallon JT. In situ localization of tissue factor in human atherosclerotic plaques by binding of digoxigenin-labeled factors VIIa and X. Laboratory investigation; a journal of technical methods and pathology 1996;75:451-61.

76. Stojkovic S, Kaun C, Basilio J, et al. Tissue factor is induced by interleukin-33 in human endothelial cells: a new link between coagulation and inflammation. Scientific reports 2016;6:25171.

77. Lin MC, Almus-Jacobs F, Chen HH, et al. Shear stress induction of the tissue factor gene. The Journal of clinical investigation 1997;99:737-44.

78. Bevilacqua MP, Pober JS, Majeau GR, Fiers W, Cotran RS, Gimbrone MA, Jr. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. Proceedings of the National Academy of Sciences of the United States of America 1986;83:4533-7.

79. Galdal KS, Lyberg T, Evensen SA, Nilsen E, Prydz H. Thrombin induces thromboplastin synthesis in cultured vascular endothelial cells. Thrombosis and haemostasis 1985;54:373-6.

80. Miller DL, Yaron R, Yellin MJ. CD40L-CD40 interactions regulate endothelial cell surface tissue factor and thrombomodulin expression. Journal of leukocyte biology 1998;63:373-9.

81. Drake TA, Hannani K, Fei HH, Lavi S, Berliner JA. Minimally oxidized low-density lipoprotein induces tissue factor expression in cultured human endothelial cells. The American journal of pathology 1991;138:601-7.

82. Carlsen E, Flatmark A, Prydz H. Cytokine-induced procoagulant activity in monocytes and endothelial cells. Further enhancement by cyclosporine. Transplantation 1988;46:575-80.

83. Mach F, Schonbeck U, Bonnefoy JY, Pober JS, Libby P. Activation of monocyte/macrophage functions related to acute atheroma complication by ligation of CD40: induction of collagenase, stromelysin, and tissue factor. Circulation 1997;96:396-9.

84. Schonbeck U, Mach F, Sukhova GK, et al. CD40 ligation induces tissue factor expression in human vascular smooth muscle cells. The American journal of pathology 2000;156:7-14.

85. Ruf W, Dickinson CD. Allosteric regulation of the cofactor-dependent serine protease coagulation factor VIIa. Trends in cardiovascular medicine 1998;8:350-6.

86. Versteeg HH, Heemskerk JW, Levi M, Reitsma PH. New fundamentals in hemostasis. Physiological reviews 2013;93:327-58.

87. Rao LV, Bajaj SP, Rapaport SI. Activation of human factor VII during clotting in vitro. Blood 1985;65:218-26.

88. Neuenschwander PF, Fiore MM, Morrissey JH. Factor VII autoactivation proceeds via interaction of distinct protease-cofactor and zymogen-cofactor complexes. Implications of a two-dimensional enzyme kinetic mechanism. The Journal of biological chemistry 1993;268:21489-92.

89. Monroe DM, Hoffman M, Roberts HR. Transmission of a procoagulant signal from tissue factor-bearing cell to platelets. Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis 1996;7:459-64.

90. Monroe DM, Hoffman M. What does it take to make the perfect clot? Arteriosclerosis, thrombosis, and vascular biology 2006;26:41-8.

91. Davie EW, Fujikawa K, Kisiel W. The coagulation cascade: initiation, maintenance, and regulation. Biochemistry 1991;30:10363-70.

92. Coughlin SR. Thrombin signalling and protease-activated receptors. Nature 2000;407:258-64.

93. Girard TJ, Warren LA, Novotny WF, et al. Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor. Nature 1989;338:518-20.

94. Ameri A, Kuppuswamy MN, Basu S, Bajaj SP. Expression of tissue factor pathway inhibitor by cultured endothelial cells in response to inflammatory mediators. Blood 1992;79:3219-26.

95. Huang ZF, Wun TC, Broze GJ, Jr. Kinetics of factor Xa inhibition by tissue factor pathway inhibitor. The Journal of biological chemistry 1993;268:26950-5.

96. Day SM, Reeve JL, Pedersen B, et al. Macrovascular thrombosis is driven by tissue factor derived primarily from the blood vessel wall. Blood 2005;105:192-8.

97. Pawashe AB, Golino P, Ambrosio G, et al. A monoclonal antibody against rabbit tissue factor inhibits thrombus formation in stenotic injured rabbit carotid arteries. Circulation research 1994;74:56-63.

98. Matetzky S, Tani S, Kangavari S, et al. Smoking increases tissue factor expression in atherosclerotic plaques: implications for plaque thrombogenicity. Circulation 2000;102:602-4.

99. Felmeden DC, Spencer CG, Chung NA, et al. Relation of thrombogenesis in systemic hypertension to angiogenesis and endothelial damage/dysfunction (a substudy of the Anglo-Scandinavian Cardiac Outcomes Trial [ASCOT]). The American journal of cardiology 2003;92:400-5.

100. Lim HS, Blann AD, Lip GY. Soluble CD40 ligand, soluble P-selectin, interleukin-6, and tissue factor in diabetes mellitus: relationships to cardiovascular disease and risk factor intervention. Circulation 2004;109:2524-8.

101. Sambola A, Osende J, Hathcock J, et al. Role of risk factors in the modulation of tissue factor activity and blood thrombogenicity. Circulation 2003;107:973-7.

102. Suefuji H, Ogawa H, Yasue H, et al. Increased plasma tissue factor levels in acute myocardial infarction. American heart journal 1997;134:253-9.

103. Reuter S, Lang D. Life span of monocytes and platelets: importance of interactions. Frontiers in bioscience 2009;14:2432-47.

104. Newman PJ, Gorski J, White GC, 2nd, Gidwitz S, Cretney CJ, Aster RH. Enzymatic amplification of platelet-specific messenger RNA using the polymerase chain reaction. The Journal of clinical investigation 1988;82:739-43.

105. Kieffer N, Guichard J, Farcet JP, Vainchenker W, Breton-Gorius J. Biosynthesis of major platelet proteins in human blood platelets. European journal of biochemistry 1987;164:189-95.

106. Marcus AJ, Safier LB, Hajjar KA, et al. Inhibition of platelet function by an aspirin-insensitive endothelial cell ADPase. Thromboregulation by endothelial cells. The Journal of clinical investigation 1991;88:1690-6.

107. Ruggeri ZM, Mendolicchio GL. Adhesion mechanisms in platelet function. Circulation research 2007;100:1673-85.

108. Ruggeri ZM. Structure and function of von Willebrand factor. Thrombosis and haemostasis 1999;82:576-84.

109. Kaplan ZS, Jackson SP. The role of platelets in atherothrombosis. Hematology / the Education Program of the American Society of Hematology American Society of Hematology Education Program 2011;2011:51-61.

110. Gachet C. ADP receptors of platelets and their inhibition. Thrombosis and haemostasis 2001;86:222-32.

111. Jin J, Daniel JL, Kunapuli SP. Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. The Journal of biological chemistry 1998;273:2030-4.

112. Hollopeter G, Jantzen HM, Vincent D, et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 2001;409:202-7.

113. Burnstock G. Purine and pyrimidine receptors. Cellular and molecular life sciences : CMLS 2007;64:1471-83.

114. Kauffenstein G, Bergmeier W, Eckly A, et al. The P2Y(12) receptor induces platelet aggregation through weak activation of the alpha(IIb)beta(3) integrin--a phosphoinositide 3-kinase-dependent mechanism. FEBS letters 2001;505:281-90.

115. Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. Proceedings of the National Academy of Sciences of the United States of America 1998;95:8070-4.

116. Nylander S, Mattsson C, Ramstrom S, Lindahl TL. Synergistic action between inhibition of P2Y12/P2Y1 and P2Y12/thrombin in ADP- and thrombin-induced human platelet activation. British journal of pharmacology 2004;142:1325-31.

117. Boersma E, Harrington RA, Moliterno DJ, et al. Platelet glycoprotein IIb/IIIa inhibitors in acute coronary syndromes: a meta-analysis of all major randomised clinical trials. Lancet 2002;359:189-98.

118. Takayama H, Hosaka Y, Nakayama K, et al. A novel antiplatelet antibody therapy that induces cAMP-dependent endocytosis of the GPVI/Fc receptor gamma-chain complex. The Journal of clinical investigation 2008;118:1785-95.

119. Fontayne A, Meiring M, Lamprecht S, et al. The humanized anti-glycoprotein Ib monoclonal antibody h6B4-Fab is a potent and safe antithrombotic in a high shear arterial thrombosis model in baboons. Thrombosis and haemostasis 2008;100:670-7.

120. Gaussem P, Reny JL, Thalamas C, et al. The specific thromboxane receptor antagonist S18886: pharmacokinetic and pharmacodynamic studies. Journal of thrombosis and haemostasis : JTH 2005;3:1437-45.

121. Wallentin L, Becker RC, Budaj A, et al. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. The New England journal of medicine 2009;361:1045-57.

122. Wiviott SD, Braunwald E, McCabe CH, et al. Prasugrel versus clopidogrel in patients with acute coronary syndromes. The New England journal of medicine 2007;357:2001-15.

123. Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. The New England journal of medicine 2001;345:494-502.

124. Bhatt DL, Stone GW, Mahaffey KW, et al. Effect of platelet inhibition with cangrelor during PCI on ischemic events. The New England journal of medicine 2013;368:1303-13.

125. Love BB, Biller J, Gent M. Adverse haematological effects of ticlopidine. Prevention, recognition and management. Drug safety 1998;19:89-98.

126. Nylander S, Schulz R. Effects of P2Y12 receptor antagonists beyond platelet inhibition--comparison of ticagrelor with thienopyridines. British journal of pharmacology 2016;173:1163-78.

127. Wallentin L. P2Y(12) inhibitors: differences in properties and mechanisms of action and potential consequences for clinical use. European heart journal 2009;30:1964-77.

128. Aleil B, Ravanat C, Cazenave JP, Rochoux G, Heitz A, Gachet C. Flow cytometric analysis of intraplatelet VASP phosphorylation for the detection of clopidogrel resistance in

patients with ischemic cardiovascular diseases. Journal of thrombosis and haemostasis : JTH 2005;3:85-92.

129. Bonello L, Tantry US, Marcucci R, et al. Consensus and future directions on the definition of high on-treatment platelet reactivity to adenosine diphosphate. Journal of the American College of Cardiology 2010;56:919-33.

130. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, et al. Contribution of gene sequence variations of the hepatic cytochrome P450 3A4 enzyme to variability in individual responsiveness to clopidogrel. Arteriosclerosis, thrombosis, and vascular biology 2006;26:1895-900.

131. Collet JP, Cuisset T, Range G, et al. Bedside monitoring to adjust antiplatelet therapy for coronary stenting. The New England journal of medicine 2012;367:2100-9.

132. Jernberg T, Payne CD, Winters KJ, et al. Prasugrel achieves greater inhibition of platelet aggregation and a lower rate of non-responders compared with clopidogrel in aspirin-treated patients with stable coronary artery disease. European heart journal 2006;27:1166-73.

133. Wiviott SD, Trenk D, Frelinger AL, et al. Prasugrel compared with high loading- and maintenance-dose clopidogrel in patients with planned percutaneous coronary intervention: the Prasugrel in Comparison to Clopidogrel for Inhibition of Platelet Activation and Aggregation-Thrombolysis in Myocardial Infarction 44 trial. Circulation 2007;116:2923-32.

134. Sugidachi A, Ogawa T, Kurihara A, et al. The greater in vivo antiplatelet effects of prasugrel as compared to clopidogrel reflect more efficient generation of its active metabolite with similar antiplatelet activity to that of clopidogrel's active metabolite. Journal of thrombosis and haemostasis : JTH 2007;5:1545-51.

135. Gurbel PA, Bliden KP, Butler K, et al. Randomized double-blind assessment of the ONSET and OFFSET of the antiplatelet effects of ticagrelor versus clopidogrel in patients with stable coronary artery disease: the ONSET/OFFSET study. Circulation 2009;120:2577-85.

136. Kuijpers MJ, Megens RT, Nikookhesal E, et al. Role of newly formed platelets in thrombus formation in rat after clopidogrel treatment: comparison to the reversible binding P2Y(1)(2) antagonist ticagrelor. Thrombosis and haemostasis 2011;106:1179-88.

137. Ibrahim H, Schutt RC, Hannawi B, DeLao T, Barker CM, Kleiman NS. Association of immature platelets with adverse cardiovascular outcomes. Journal of the American College of Cardiology 2014;64:2122-9.

138. Hoefer T, Armstrong PC, Finsterbusch M, Chan MV, Kirkby NS, Warner TD. Drug-Free Platelets Can Act as Seeds for Aggregate Formation During Antiplatelet Therapy. Arteriosclerosis, thrombosis, and vascular biology 2015;35:2122-33.

139. Bonaca MP, Bhatt DL, Cohen M, et al. Long-Term Use of Ticagrelor in Patients with Prior Myocardial Infarction. The New England journal of medicine 2015.

140. Adamski P, Kozinski M, Ostrowska M, et al. Overview of pleiotropic effects of platelet P2Y12receptor inhibitors. Thrombosis and haemostasis 2014;112:224-42.

141. Takeda M, Yamashita T, Shinohara M, et al. Beneficial effect of anti-platelet therapies on atherosclerotic lesion formation assessed by phase-contrast X-ray CT imaging. The international journal of cardiovascular imaging 2012;28:1181-91.

142. Totani L, Dell'Elba G, Martelli N, et al. Prasugrel inhibits platelet-leukocyte interaction and reduces inflammatory markers in a model of endotoxic shock in the mouse. Thrombosis and haemostasis 2012;107:1130-40.

143. Froldi G, Bertin R, Dorigo P, Montopoli M, Caparrotta L. Endothelium-independent vasorelaxation by ticlopidine and clopidogrel in rat caudal artery. The Journal of pharmacy and pharmacology 2011;63:1056-62.

144. Warnholtz A, Ostad MA, Velich N, et al. A single loading dose of clopidogrel causes dose-dependent improvement of endothelial dysfunction in patients with stable coronary artery disease: results of a double-blind, randomized study. Atherosclerosis 2008;196:689-95.

145. Cattaneo M, Schulz R, Nylander S. Adenosine-mediated effects of ticagrelor: evidence and potential clinical relevance. Journal of the American College of Cardiology 2014;63:2503-9.

146. Armstrong D, Summers C, Ewart L, Nylander S, Sidaway JE, van Giezen JJ. Characterization of the adenosine pharmacology of ticagrelor reveals therapeutically relevant inhibition of equilibrative nucleoside transporter 1. Journal of cardiovascular pharmacology and therapeutics 2014;19:209-19.

147. King AE, Ackley MA, Cass CE, Young JD, Baldwin SA. Nucleoside transporters: from scavengers to novel therapeutic targets. Trends in pharmacological sciences 2006;27:416-25.

148. van Giezen JJ, Sidaway J, Glaves P, Kirk I, Bjorkman JA. Ticagrelor inhibits adenosine uptake in vitro and enhances adenosine-mediated hyperemia responses in a canine model. Journal of cardiovascular pharmacology and therapeutics 2012;17:164-72.

149. Bonello L, Laine M, Kipson N, et al. Ticagrelor increases adenosine plasma concentration in patients with an acute coronary syndrome. Journal of the American College of Cardiology 2014;63:872-7.

150. Johnston-Cox HA, Yang D, Ravid K. Physiological implications of adenosine receptor-mediated platelet aggregation. Journal of cellular physiology 2011;226:46-51.

151. Nylander S, Femia EA, Scavone M, et al. Ticagrelor inhibits human platelet aggregation via adenosine in addition to P2Y12 antagonism. Journal of thrombosis and haemostasis : JTH 2013;11:1867-76.

152. Grzesk G, Kozinski M, Navarese EP, et al. Ticagrelor, but not clopidogrel and prasugrel, prevents ADP-induced vascular smooth muscle cell contraction: a placebo-controlled study in rats. Thrombosis research 2012;130:65-9.

153. Rahman M, Gustafsson D, Wang Y, Thorlacius H, Braun OO. Ticagrelor reduces neutrophil recruitment and lung damage in abdominal sepsis. Platelets 2013.

154. Nanhwan MK, Ling S, Kodakandla M, Nylander S, Ye Y, Birnbaum Y. Chronic treatment with ticagrelor limits myocardial infarct size: an adenosine and cyclooxygenase-2-dependent effect. Arteriosclerosis, thrombosis, and vascular biology 2014;34:2078-85.

155. Vilahur G, Gutierrez M, Casani L, et al. Protective Effects of Ticagrelor on Myocardial Injury After Infarction. Circulation 2016;134:1708-19.

156. Torngren K, Ohman J, Salmi H, Larsson J, Erlinge D. Ticagrelor improves peripheral arterial function in patients with a previous acute coronary syndrome. Cardiology 2013;124:252-8.

157. Furie B, Furie BC. Mechanisms of thrombus formation. The New England journal of medicine 2008;359:938-49.

158. Furie B, Furie BC. Thrombus formation in vivo. The Journal of clinical investigation 2005;115:3355-62.

159. Libby P. Mechanisms of acute coronary syndromes and their implications for therapy. The New England journal of medicine 2013;368:2004-13.

160. Jackson SP. Arterial thrombosis--insidious, unpredictable and deadly. Nature medicine 2011;17:1423-36.

161. Stone GW, Maehara A, Lansky AJ, et al. A prospective natural-history study of coronary atherosclerosis. The New England journal of medicine 2011;364:226-35.

162. Motoyama S, Sarai M, Harigaya H, et al. Computed tomographic angiography characteristics of atherosclerotic plaques subsequently resulting in acute coronary syndrome. Journal of the American College of Cardiology 2009;54:49-57.

163. Davies MJ, Richardson PD, Woolf N, Katz DR, Mann J. Risk of thrombosis in human atherosclerotic plaques: role of extracellular lipid, macrophage, and smooth muscle cell content. British heart journal 1993;69:377-81.

164. Glagov S, Weisenberg E, Zarins CK, Stankunavicius R, Kolettis GJ. Compensatory enlargement of human atherosclerotic coronary arteries. The New England journal of medicine 1987;316:1371-5.

165. Libby P, Theroux P. Pathophysiology of coronary artery disease. Circulation 2005;111:3481-8.

166. Yonetsu T, Kakuta T, Lee T, et al. In vivo critical fibrous cap thickness for rupture-prone coronary plaques assessed by optical coherence tomography. European heart journal 2011;32:1251-9.

167. Tang TY, Howarth SP, Miller SR, et al. The ATHEROMA (Atorvastatin Therapy: Effects on Reduction of Macrophage Activity) Study. Evaluation using ultrasmall superparamagnetic iron oxide-enhanced magnetic resonance imaging in carotid disease. Journal of the American College of Cardiology 2009;53:2039-50.

168. Nidorf SM, Eikelboom JW, Budgeon CA, Thompson PL. Low-dose colchicine for secondary prevention of cardiovascular disease. Journal of the American College of Cardiology 2013;61:404-10.

169. Ariens RA, Lai TS, Weisel JW, Greenberg CS, Grant PJ. Role of factor XIII in fibrin clot formation and effects of genetic polymorphisms. Blood 2002;100:743-54.

170. Rollini F, Franchi F, Cho JR, et al. A head-to-head pharmacodynamic comparison of prasugrel vs. ticagrelor after switching from clopidogrel in patients with coronary artery disease: results of a prospective randomized study. European heart journal 2016;37:2722-30.

171. Wihlborg AK, Wang L, Braun OO, et al. ADP receptor P2Y12 is expressed in vascular smooth muscle cells and stimulates contraction in human blood vessels. Arteriosclerosis, thrombosis, and vascular biology 2004;24:1810-5.

5 Original Articles

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Ticagrelor, but not Clopidogrel, Reduces Arterial Thrombosis via Endothelial Tissue Factor Suppression

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Ticagrelor, but not Clopidogrel Active Metabolite, Displays Antithrombotic Properties in the Left Atrial Endocardium

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6 Summary

6.1 Ticagrelor, but not clopidogrel active metabolite, reduces endothelial tissue factor via proteasomal degradation

In our initial study we showed that ticagrelor, unlike CAM, dose-dependently reduced TF protein expression as well as TF enzyme activity in TNF-α-stimulated HAECs indicating local antithrombotic properties of ticagrelor on the human endothelium[1] in addition to its well-described anti-platelet effects.[2] By investigating the underlying molecular mechanisms, we found that the reduction in endothelial TF expression by ticagrelor was reversed by wortmannin and rapamycin suggesting the involvement of the signalling molecules PI3K and p70s6 kinase.[1] Furthermore, inhibition of proteasomes using MG-132 reversed the effects of ticagrelor on TF expression in HAECs.[1] On the other hand, ticagrelor did not decrease TF mRNA nor did it affect TF mRNA stability indicating that ticagrelor reduced endothelial TF expression by proteasomal degradation rather than mRNA modification.[1] Interestingly, the observed effects of ticagrelor were mediated independently of the ADP receptor $P2Y_{12}$,[1] the target receptor of ticagrelor on platelets[3], since it was not expressed in HAECs, neither on the mRNA nor on the protein level.[1] Recently it was shown that ticagrelor also inhibits the adenosine transporter ENT1,[4] which mediates cellular uptake of adenosine in red blood cells, among others,[5] thereby increasing adenosine plasma levels in patients treated with ticagrelor.[6] These findings led to the hypothesis that ticagrelor may inhibit ENT1, known to be expressed in endothelial cells,[7] and subsequently increases extracellular adenosine levels, which in turn affect TF expression through one of the four endothelial adenosine receptors[8]. In line with previous reports,[9] adenosine dose-dependently decreased TF expression in TNF-α-stimulated HAECs.[1] However, adenosine, unlike ticagrelor, exerted its effect via a reduction of TF mRNA.[1] In contrast to ticagrelor, dipyridamole, a slightly more potent ENT1 inhibitor[4], did not reduce TF protein expression in HAECs and thus, not mimic the effects of ticagrelor.[1] Lastly, inhibition of all adenosine receptors individually or in combination did not reverse the

reduction of TF mediated by ticagrelor thereby disproving the ENT1 hypothesis in endothelial cells.[1]

6.2 Ticagrelor, compared with clopidogrel, decreases endothelial tissue factor expression and arterial thrombosis in mice

Next, we investigated the physiological relevance of our *in vitro* findings in a mouse model of photochemical-induced arterial thrombosis and found that ticagrelor, but not clopidogrel, at physiological plasma concentrations, reduced TF expression in the endothelium of common carotid arteries.[1] Furthermore, ticagrelor prolonged time to arterial occlusion, compared with clopidogrel, in mice.[1] Plasma TF activity and endogenous thrombin potential, on the other hand, were comparable among the treated groups.[1] Likewise, platelet aggregation in response to ADP was inhibited to similar extents in both mice treated with ticagrelor and clopidogrel.[1] These findings indicate that ticagrelor exerts more potent antithrombotic properties, compared with clopidogrel, which was associated with a local reduction of endothelial TF expression and independent of changes in plasma TF activity, systemic coagulation or inhibition of ADP-induced platelet aggregation.[1]

6.3 Ticagrelor, unlike clopidogrel active metabolite, reduces thrombogenicity in atrial fibrillation patients

AF triggers thrombus formation in LAA leading to systemic embolism such as ischemic stroke causing substantial morbidity and mortality.[10] Although anticoagulant treatment is considered standard therapy to prevent thromboembolic complications[10], patients with AF frequently have comorbidities such as ACS requiring antiplatelet therapy.[11] Therefore, we studied whether certain $P2Y_{12}$ receptor antagonist may possess local antithrombotic properties in endocardial cells isolated from LAA of AF patients.[12] Recently it was shown that the thrombotic factors TF and plasminogen activator inhibitor-1 (PAI-1) are expressed to a higher extent in left, as compared with right atrial appendages, which may in part explain the greater

thrombogenicity observed in LAA.[13] In accordance with our previous findings in HAECs and a mouse model of arterial thrombosis[1] we found that ticagrelor, unlike CAM, reduced TF and PAI-1 protein expressions as well as enzyme activities in TNF-α-stimulated LAA endocardial cells isolated from AF patients.[12] On the other hand we did not observe different expression levels of TFPI, the physiological antagonist of TF.[12] Indeed, this translational study indicates local antithrombotic properties of ticagrelor, which may lower thrombogenicity in AF patients.[12]

7 Discussion

7.1 Ticagrelor exhibits platelet-independent antithrombotic effects on the endothelium

Cardiovascular disease including coronary artery disease and stroke among others is the leading cause of death in both men and women in Europe.[14] Arterial thrombus formation following rupture of an atherosclerotic plaque is the key pathophysiological mechanisms leading to an ACS.[15] Platelets, in addition to the coagulation system, are crucially involved in thrombus formation and inhibition of platelet aggregation has been proven to reduce thrombogenesis and thus MI and stroke.[16-19] In 1988 the second international study of infarct survival (ISIS-2) study showed that platelet inhibition by aspirin reduces non-fatal MI, non-fatal stroke and vascular mortality, compared with placebo.[16] Later, dual antiplatelet therapy (DAPT) using the $P2Y_{12}$ receptor antagonist clopidogrel in addition to aspirin was reported to further reduce non-fatal MI, stroke and CV death in the Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) study.[17] In more recent years, however, newer generations of $P2Y_{12}$ receptor antagonists have evolved and were compared with the thienopyridine clopidogrel.[18,19] Prasugrel is a third generation thienopyridine and like clopidogrel requires cytochrome P450 metabolism in order to form its active metabolite, which irreversibly binds the platelet ADP receptor $P2Y_{12}$.[2] Yet, prasugrel only requires a one-step activation, as compared with the two-step activation of clopidogrel[20] and platelet inhibition occurs faster and to a higher extend; also, inter-individual variability and non-responsiveness to prasugrel is lower.[21,22] Consequently, prasugrel reduced the incidence of death from cardiovascular causes, non-fatal MI, or non-fatal stroke in patients with ACS, as compared with clopidogrel.[19] Nevertheless, prasugrel also increased major bleeding rates and thus, overall mortality was comparable in prasugrel- and clopidogrel treated patients.[19] In contrast, ticagrelor is a cyclopentyl-triazolo-pyrimidine, which does not require cytochrome P450 activation and binds the $P2Y_{12}$ receptor directly and reversibly[2]. Like prasugrel, ticagrelor displays faster and greater inhibition of ADP-induced platelet aggregation[23] and

inhibits newly formed platelets more efficiently in patients with high platelet turnover[24]. Correspondingly, ticagrelor, compared with clopidogrel, further reduced the incidence of death from vascular causes, MI and stroke in ACS patients in the Platelet Inhibition and Patient Outcomes (PLATO) study.[18] Importantly, major bleedings were not higher and ticagrelor reduced overall mortality.[18] Despite the fact that both prasugrel and ticagrelor show greater platelet inhibition compared with clopidogrel,[2] only ticagrelor reduced overall mortality[18,22]. Therefore, we hypothesized that platelet-independent effects of ticagrelor may contribute to this observation.

Indeed, we found that ticagrelor reduced endothelial TF expression and activity by proteasomal degradation in HAECs and that ticagrelor decreased endothelial TF expression in murine arteries subsequently prolonging time to arterial occlusion in mice, compared with clopidogrel.[1] Furthermore, ticagrelor lowered TF and PAI-1 expression in endocardial cells isolated from LAA of AF patients.[12] These results strongly indicate local antithrombotic properties of ticagrelor on endothelial and endocardial cells and may in part explain the reduced mortality observed in PLATO.

7.2 Ticagrelor-mediated tissue factor reduction in endothelial cells and its underlying molecular mechanisms

We found that ticagrelor, but not CAM, concentration-dependently reduced TF expression as well as TF activity in HAECs and subsequently investigated the underlying molecular mechanisms.[1] We found that the signalling molecules PI3K and p70s6 were involved in the reduction of TF protein expression by ticagrelor.[1] This observation pointed towards an involvement of the $P2Y_{12}$ receptor, a metabotropic G-protein coupled receptor linked to the downstream signalling molecule PI3K in platelets[25]. Nevertheless, we did not detected $P2Y_{12}$ mRNA or protein in endothelial cells.[1]

Recently, platelet- as well as $P2Y_{12}$ receptor-independent pleiotropic effects have been described for ticagrelor.[26] Unlike thienopyridines, ticagrelor also binds to the adenosine

transporter ENT1,[4] which is expressed in red blood cells and endothelial cells among others.[5,7] ENT1 mediates adenosine uptake in red blood cells, which was decreased by ticagrelor *in vitro*[27] and correspondingly, higher adenosine plasma levels were found in ACS patients after treatment with ticagrelor.[6] Adenosine is known to inhibit platelet aggregation[28] and indeed, adenosine contributed to the inhibition platelet aggregation in whole blood treated with ticagrelor[29]. Since both ENT1 and adenosine receptors are expressed in endothelial cells[7,8], we tested the hypothesis that ticagrelor inhibits ENT1 in HAECS increasing extracellular levels of adenosine and subsequently reducing TF via endothelial adenosine receptors. Indeed, adenosine decreased endothelial TF expression; however; this observation was mediated via a reduction in TF mRNA rather than proteasomal degradation as observed with ticagrelor[1]. Also, the ENT1 inhibitor dipyridamole[4] did not mimic, and inhibition of adenosine receptors did not reverse the effects of ticagrelor on TF expression suggesting that our observations were occurring independently of ENT1.[1] Indeed, ticagrelor shows side effects similar to adenosine such as dyspnoea[18,30,31] and increased adenosine levels through inhibition of ENT1 by ticagrelor[4] represent a plausible explanation for this finding. Nevertheless, several arguments speak against this hypothesis. Firstly, the ENT1 inhibitor dipyridamole does not cause dyspnoea[32,33] and secondly, the adenosine triphosphate analogue cangrelor, which, like ticagrelor, inhibits $P2Y_{12}$ receptors reversibly but does not bind to ENT1[4] also causes dyspnoea, albeit to a lesser extent.[34,35] These observation further support the concept that ticagrelor may exert additional mechanism beyond $P2Y_{12}$ and ENT1 inhibition.

Other molecular mechanisms potentially explaining our *in vitro* findings include direct binding of ticagrelor to adenosine receptors due to their molecular similarity. Indeed, ticagrelor shows low binding affinity to the adenosine receptors A_1, A_{2a}, A_{2b} and A_3; however clinical concentrations of ticagrelor are of neglectable functional relevance.[4] Moreover, ticagrelor may exhibit binding affinity to other family members of the P2 purine and pyrimidine

receptors expressed in endothelial cells.[36] However, to date no such interactions have been described.

7.3 Ticagrelor and arterial thrombosis *in vivo* – relevance of endothelial tissue factor

In C57BL/6 mice we found that ticagrelor reduced arterial thrombosis more effectively than clopidogrel and, correspondingly, that ticgrelor-treated mice showed lower endothelial TF expression.[1] On the other hand, we did not observe differences in platelet aggregation or systemic coagulation including plasma TF activity and endogenous thrombin potential between the two groups.[1] These results suggest that ticagrelor exerts local antithrombotic properties by reducing endothelial TF expression in addition to its well-described antiplatelet effects.[1]

TF plays a crucial role in arterial thrombosis as it initiates the extrinsic coagulation cascade by activating factor VII[37] followed by factor IX and X[38] and subsequently thrombin; thrombin in turn, activates a positive feedback loop[39] finally leading to large amounts of fibrin formation[40] in addition to platelet activation.[41] TF is expressed in the entire vasculature including endothelial cells[42], VSMC and adventitial cells[43] as well as on circulating TF-containing microparticles released from endothelial cells[44]; In pathological conditions, such as high shear flow[45] as occurring in a stenosed artery and during inflammation as occurring in atherosclerosis, TF expression is upregulated.[46] Consequently, TF is detected in the necrotic core of atherosclerotic plaques.[43,47,48] Vascular, rather than blood cell-derived TF appears to play a relevant role in arterial thrombus formation.[49] Day and colleagues showed that mice expressing low amounts of TF in bone marrow cells did not have prolonged arterial occlusion times and that mice with TF deficiency that were transplanted with bone marrow cells expressing physiologic amounts of TF, did not have reduced arterial occlusion times.[49] Importantly, the group used the same experimental mouse model for arterial thrombosis as we did in our study, i.e. photochemical injury of the carotid artery by bengal rose.[49] The

relevance of TF in arterial thrombosis was further supported by Pawashe et al. showing that anti-TF antibody treatment decreased arterial thrombosis in common carotid arteries of rabbits[50]. Lastly, clinical studies found an association of TF with cardiovascular risk factors[51-54] and the incidence of MI.[55]

Whether indeed the decrease of endothelial TF expression by ticagrelor entirely explains the reduction in arterial thrombosis observed in mice remains to be proven by rescue experiments. Since the exact mechanisms leading to TF degradation are not entirely understood, it appears difficult to rescue endothelial TF reduction *in vivo* without affecting other sources of TF. On the other hand, one would expect no differences in arterial thrombus formation in endothelial-specific TF knockout mice treated with ticagrelor or clopidogrel and exposed to photochemical injury of the common carotid arteries. Yet, we did not have access to such a mouse strain.

Although we have ruled out other potential and relevant mechanisms explaining the differences in arterial thrombus formation between ticagrelor- and clopidogrel-treated rodents, such as alterations in platelet aggregation, plasma TF activity and systemic coagulation, additional possible explanations remain. Kirby et al. reported that inhibition of platelet aggregation by NO, which is usually rather low, increased significantly in the presence of the $P2Y_{12}$ receptor antagonist prasugrel active metabolite or ticagrelor.[56] This finding was explained by synergistic effects of NO and $P2Y_{12}$ receptor inhibition on the reduction of the downstream signalling molecule PI3K.[56] NO is produced in endothelial cells by endothelial NO synthase[57] and inducible NO synthase during inflammation[58]. Interestingly, we have previously shown that ticagrelor, unlike CAM, dose-dependently increases phosphorylation of endothelial NO synthase at the activation side serine 1177.[59] In line with our findings, treatment with ticagrelor augmented myocardial NO synthase in rats.[60] Increased phosphorylation of endothelial NO synthase may result in higher concentration of NO at the vessel wall and may contribute to higher platelet inhibition *in vivo.*

Drug dosages of P2Y12 receptor antagonists in rodents

In humans, ticagrelor, compared with clopidogrel, exerts greater inhibition of ADP-induced platelet aggregation[23]. Therefore, different effects on MI, stroke and CV death between clopidogrel- and ticagrelor-treated patients[18,30] may be due to different platelet inhibitory effects. To rule out platelet-dependent effects in our study we selected dosages of ticagrelor and clopidogrel that resulted in comparable inhibition of ADP-induced platelet aggregation.[1] Importantly, we performed platelet aggregometry in whole blood as it was recently reported that adenosine contributes to whole blood platelet inhibition in the presence of ticagrelor.[29] In order to show that our experiments were performed at minimal drug concentrations and that none of the drugs were used at excessively high concentrations, we performed dose response experiments and found that reducing drug dosages by only one third resulted in residual and comparable platelet reactivity in ticagrelor- and clopidogrel-treated animals.[1] Besides, dosages in our *in vitro* and *in vivo* experiments were chosen so as to reflect plasma concentration found in humans. Recommended dosages in cardiovascular patients for clopidogrel and ticagrelor represent a loading dosage of 300 – 600 mg followed by 75 mg once daily and a loading dosage of 180 mg followed by 90 mg twice daily, respectively.[11] Thus, maintenance dosages differ 2.4 fold. Such dosages provide plasma concentration of 0.16 – 0.18 uM of clopidogrel active metabolite and 1 – 1.5 uM of ticagrelor in humans[2]. Likewise, drug dosages in our study differed 2.5 fold (ticagrelor 0.15% vs. clopidogrel 0.06%).[1] However, unlike in humans,[23] these dosages resulted in equivalent platelet inhibition in mice[1], which was also in line with previous findings in rats.[60] In addition, we evaluated ticagrelor plasma concentration in mice and found concentrations of 2.7±0.7 uM,[1] which were comparable to humans.[2]

8 Outlook

Here we report that ticagrelor, unlike CAM, reduces endothelial TF by proteasomal degradation *in vitro*.[1] On a molecular level we found that the effect of ticagrelor on TF was mediated by the signalling pathways PI3K and p70s6 kinase.[1] We have shown that the observed effects were independent of the $P2Y_{12}$ receptor as well as ENT1 indicating that other mechanisms may be involved.[1] Yet, specific additional target receptors of ticagrelor remain to be determined. To assess the physiological relevance of our *in vitro* data *in vivo*, we showed that ticagrelor, unlike clopidogrel, reduced endothelial TF expression in common carotid arteries and prolonged arterial occlusion times in mice; further, we ruled out other potential and relevant mechanisms, which could have contributed to this observation including platelet aggregation, plasma TF activity and systemic coagulation.[1] In order to finally prove the relevance of endothelial TF in our experiments, a rescue experiment specifically preventing the reduction of endothelial TF by ticagrelor *in vivo*, without affecting other sources of TF, appears essential.

Our study indicates local antithrombotic effects of ticagrelor at the vessel wall in addition to its antiplatelet effects.[1] Such properties may have contributed to the reduction of clinical events including MI and stroke in ACS patients treated with ticagrelor in clinical trials.[18,30] On the other hand, dual antithrombotic effects of ticagrelor may contribute to an increased bleeding risk. Although ticagrelor did not augment total major bleeding events, fatal intracranial bleeds were higher in ACS patients treated with ticagrelor, compared with clopidogrel.[18] Whether this observation may be due to higher platelet inhibition[23] or due to the local antithrombotic properties of ticagrelor[1] remain speculative.

In a subsequent study we investigated whether the antithrombotic properties displayed by ticagrelor[12] could also be relevant in AF patients. Previously it was reported that LAA endocardial cells express higher levels of procoagulant TF and PAI-1, as compared with right atrial appendages, which may contribute to the higher thrombogenicity observed in LAA.[13]

Therefore, we investigated whether ticagrelor or CAM may affect TF or PAI-1 expression in LAA endocardial cells isolated from AF patients. Indeed, we found that ticagrelor, but not CAM, reduced TF and PAI-1 protein expressions as well as enzyme activities indicating local antithrombotic mechanisms in AF patients.[12] AF is a common cardiac arrhythmia, which is associated with an increased risk of stroke and mortality and oral anticoagulation is the treatment of choice to reduce thromboembolic complication.[10] Indeed, anticoagulation has been shown to be superior to antiplatelet therapy (single or DAPT).[61] Yet, newer and more potent generations of $P2Y_{12}$ receptor antagonists including ticagrelor have not been compared to oral anticoagulation in AF patients in randomized clinical trials. The described local antithrombotic properties of ticagrelor in LAAs of AF patients may be of clinical relevance and may affect thrombogenicity in these patients;[12] however, this hypothesis needs to be addressed in additional clinical studies.

Frequently, AF patients have comorbidities such as ACS and require both anticoagulant and antiplatelet therapy, referred to as triple anticoagulation.[11] In such patients, ticagrelor may be the preferred choice in order to reduce thromboembolic complications. Triple anticoagulation on the other hand increases bleeding complications substantially and choosing the ideal treatment to prevent thrombotic events without causing bleedings is challenging.[11] In patients with an ACS receiving DAPT (aspirin and a thienopyridine), small dosages of oral anticoagulants are sufficient to increased bleeding rates significantly.[62] Consequently, in patients with anticoagulant treatment undergoing percutaneous intervention requiring DAPT, single (clopidogrel) versus DAPT reduced bleeding complications significantly;[63] however, although not statistical significant, higher rates of thrombotic events have been observed in the single antiplatelet therapy group.[63] Therefore, it remains to be determined whether reducing triple antithrombotic therapy to oral anticoagulation and $P2Y_{12}$ receptor inhibition is sufficient to sustain low thrombotic events. The local anti-thrombotic properties of ticagrelor appear beneficial in AF patients requiring platelet antagonists due to comorbidities such as

ACS; however, further clinical studies are required to prove the effectiveness and safety of newer generations of $P2Y_{12}$ receptor antagonists such as ticagrelor in AF.[64]

9 Abbreviations

ACS	acute coronary syndrome
ADP	adenosine diphosphate
AF	atrial fibrillation
CAM	clopidogrel active metabolite
CURE	Clopidogrel in Unstable Angina to Prevent Recurrent Events
CV	cardiovascular
CVD	cardiovascular disease
DAPT	dual antiplatelet therapy
eNOS	endothelial nitric oxide synthase
ENT1	equilibrative nucleoside transporter 1
GP	glycoprotein
H2O2	hydrogen peroxide
HAECs	human aortic endothelial cells
ISIS-2	second international study of infarct survival
LAA	left atrial appendage
LDL	low-density lipoprotein
MI	myocardial infarction
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
O^2-	superoxide anion
OH	hydroxyl radicals
ONOO-	peroxynitrite
PAI-1	plasminogen activator inhibitor-1
PGI_2	prostacyclin
PI3K	phosphoinositide 3-kinase
PLATO	study of Platelet Inhibition and Patient Outcomes

ROS	reactive oxygen species
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TNF-α	tumor necrosis factor-alpha
VCAM-1	vascular cell adhesion molecule-1
VSMC	vascular smooth muscle cells
vWF	von Willebrand factor

10 References

1. Reiner MF, Akhmedov A, Stivala S, et al. Ticagrelor, but not clopidogrel, reduces arterial thrombosis via endothelial tissue factor suppression. Cardiovascular research 2017;113:61-9.

2. Wallentin L. P2Y(12) inhibitors: differences in properties and mechanisms of action and potential consequences for clinical use. European heart journal 2009;30:1964-77.

3. Husted S, Emanuelsson H, Heptinstall S, Sandset PM, Wickens M, Peters G. Pharmacodynamics, pharmacokinetics, and safety of the oral reversible P2Y12 antagonist AZD6140 with aspirin in patients with atherosclerosis: a double-blind comparison to clopidogrel with aspirin. European heart journal 2006;27:1038-47.

4. Armstrong D, Summers C, Ewart L, Nylander S, Sidaway JE, van Giezen JJ. Characterization of the adenosine pharmacology of ticagrelor reveals therapeutically relevant inhibition of equilibrative nucleoside transporter 1. Journal of cardiovascular pharmacology and therapeutics 2014;19:209-19.

5. King AE, Ackley MA, Cass CE, Young JD, Baldwin SA. Nucleoside transporters: from scavengers to novel therapeutic targets. Trends in pharmacological sciences 2006;27:416-25.

6. Bonello L, Laine M, Kipson N, et al. Ticagrelor increases adenosine plasma concentration in patients with an acute coronary syndrome. Journal of the American College of Cardiology 2014;63:872-7.

7. Casanello P, Torres A, Sanhueza F, et al. Equilibrative nucleoside transporter 1 expression is downregulated by hypoxia in human umbilical vein endothelium. Circulation research 2005;97:16-24.

8. Burnstock G. Purinergic Signaling in the Cardiovascular System. Circulation research 2017;120:207-28.

9. Deguchi H, Takeya H, Urano H, Gabazza EC, Zhou H, Suzuki K. Adenosine regulates tissue factor expression on endothelial cells. Thrombosis research 1998;91:57-64.

10. Camm AJ, Lip GY, De Caterina R, et al. 2012 focused update of the ESC Guidelines for the management of atrial fibrillation: an update of the 2010 ESC Guidelines for the management of atrial fibrillation. Developed with the special contribution of the European Heart Rhythm Association. European heart journal 2012;33:2719-47.

11. Roffi M, Patrono C, Collet JP, et al. 2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: Task Force for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent ST-Segment Elevation of the European Society of Cardiology (ESC). European heart journal 2016;37:267-315.

12. Reiner MF, Breitenstein A, Holy EW, et al. Ticagrelor, but not clopidogrel active metabolite, displays antithrombotic properties in the left atrial endocardium. European heart journal 2017;38:916-9.

13. Breitenstein A, Glanzmann M, Falk V, et al. Increased prothrombotic profile in the left atrial appendage of atrial fibrillation patients. International journal of cardiology 2015;185:250-5.

14. Townsend N, Wilson L, Bhatnagar P, Wickramasinghe K, Rayner M, Nichols M. Cardiovascular disease in Europe: epidemiological update 2016. European heart journal 2016;37:3232-45.

15. Libby P. Mechanisms of acute coronary syndromes and their implications for therapy. The New England journal of medicine 2013;368:2004-13.

16. ISIS-2-Collaborative-Group. Randomised trial of intravenous streptokinase, oral aspirin, both, or neither among 17,187 cases of suspected acute myocardial infarction: ISIS-2. ISIS-2 (Second International Study of Infarct Survival) Collaborative Group. Lancet 1988;2:349-60.

17. Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. The New England journal of medicine 2001;345:494-502.

18. Wallentin L, Becker RC, Budaj A, et al. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. The New England journal of medicine 2009;361:1045-57.

19. Wiviott SD, Braunwald E, McCabe CH, et al. Prasugrel versus clopidogrel in patients with acute coronary syndromes. The New England journal of medicine 2007;357:2001-15.

20. Sugidachi A, Ogawa T, Kurihara A, et al. The greater in vivo antiplatelet effects of prasugrel as compared to clopidogrel reflect more efficient generation of its active metabolite with similar antiplatelet activity to that of clopidogrel's active metabolite. Journal of thrombosis and haemostasis : JTH 2007;5:1545-51.

21. Jernberg T, Payne CD, Winters KJ, et al. Prasugrel achieves greater inhibition of platelet aggregation and a lower rate of non-responders compared with clopidogrel in aspirin-treated patients with stable coronary artery disease. European heart journal 2006;27:1166-73.

22. Wiviott SD, Trenk D, Frelinger AL, et al. Prasugrel compared with high loading- and maintenance-dose clopidogrel in patients with planned percutaneous coronary intervention: the Prasugrel in Comparison to Clopidogrel for Inhibition of Platelet Activation and Aggregation-Thrombolysis in Myocardial Infarction 44 trial. Circulation 2007;116:2923-32.

23. Gurbel PA, Bliden KP, Butler K, et al. Randomized double-blind assessment of the ONSET and OFFSET of the antiplatelet effects of ticagrelor versus clopidogrel in patients with stable coronary artery disease: the ONSET/OFFSET study. Circulation 2009;120:2577-85.

24. Kuijpers MJ, Megens RT, Nikookhesal E, et al. Role of newly formed platelets in thrombus formation in rat after clopidogrel treatment: comparison to the reversible binding P2Y(1)(2) antagonist ticagrelor. Thrombosis and haemostasis 2011;106:1179-88.

25. Kauffenstein G, Bergmeier W, Eckly A, et al. The P2Y(12) receptor induces platelet aggregation through weak activation of the alpha(IIb)beta(3) integrin--a phosphoinositide 3-kinase-dependent mechanism. FEBS letters 2001;505:281-90.

26. Cattaneo M, Schulz R, Nylander S. Adenosine-mediated effects of ticagrelor: evidence and potential clinical relevance. Journal of the American College of Cardiology 2014;63:2503-9.

27. van Giezen JJ, Sidaway J, Glaves P, Kirk I, Bjorkman JA. Ticagrelor inhibits adenosine uptake in vitro and enhances adenosine-mediated hyperemia responses in a canine model. Journal of cardiovascular pharmacology and therapeutics 2012;17:164-72.

28. Johnston-Cox HA, Yang D, Ravid K. Physiological implications of adenosine receptor-mediated platelet aggregation. Journal of cellular physiology 2011;226:46-51.

29. Nylander S, Femia EA, Scavone M, et al. Ticagrelor inhibits human platelet aggregation via adenosine in addition to P2Y12 antagonism. Journal of thrombosis and haemostasis : JTH 2013;11:1867-76.

30. Bonaca MP, Bhatt DL, Cohen M, et al. Long-Term Use of Ticagrelor in Patients with Prior Myocardial Infarction. The New England journal of medicine 2015.

31. Burki NK, Lee LY. Blockade of airway sensory nerves and dyspnea in humans. Pulmonary pharmacology & therapeutics 2010;23:279-82.

32. Biaggioni I, Onrot J, Hollister AS, Robertson D. Cardiovascular effects of adenosine infusion in man and their modulation by dipyridamole. Life sciences 1986;39:2229-36.

33. Sacco RL, Diener HC, Yusuf S, et al. Aspirin and extended-release dipyridamole versus clopidogrel for recurrent stroke. The New England journal of medicine 2008;359:1238-51.

34. Bhatt DL, Stone GW, Mahaffey KW, et al. Effect of platelet inhibition with cangrelor during PCI on ischemic events. The New England journal of medicine 2013;368:1303-13.

35. Harrington RA, Stone GW, McNulty S, et al. Platelet inhibition with cangrelor in patients undergoing PCI. The New England journal of medicine 2009;361:2318-29.

36. Burnstock G. Purine and pyrimidine receptors. Cellular and molecular life sciences : CMLS 2007;64:1471-83.

37. Spicer EK, Horton R, Bloem L, et al. Isolation of cDNA clones coding for human tissue factor: primary structure of the protein and cDNA. Proceedings of the National Academy of Sciences of the United States of America 1987;84:5148-52.

38. Osterud B, Rapaport SI. Activation of factor IX by the reaction product of tissue factor and factor VII: additional pathway for initiating blood coagulation. Proceedings of the National Academy of Sciences of the United States of America 1977;74:5260-4.

39. Furie B, Furie BC. Mechanisms of thrombus formation. The New England journal of medicine 2008;359:938-49.

40. Ruf W, Edgington TS. Structural biology of tissue factor, the initiator of thrombogenesis in vivo. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 1994;8:385-90.

41. Coughlin SR. Thrombin signalling and protease-activated receptors. Nature 2000;407:258-64.

42. Zeldis SM, Nemerson Y, Pitlick FA, Lentz TL. Tissue factor (thromboplastin): localization to plasma membranes by peroxidase-conjugated antibodies. Science 1972;175:766-8.

43. Wilcox JN, Smith KM, Schwartz SM, Gordon D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. Proceedings of the National Academy of Sciences of the United States of America 1989;86:2839-43.

44. Shet AS, Aras O, Gupta K, et al. Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes. Blood 2003;102:2678-83.

45. Lin MC, Almus-Jacobs F, Chen HH, et al. Shear stress induction of the tissue factor gene. The Journal of clinical investigation 1997;99:737-44.

46. Bevilacqua MP, Pober JS, Majeau GR, Fiers W, Cotran RS, Gimbrone MA, Jr. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular

endothelium: characterization and comparison with the actions of interleukin 1. Proceedings of the National Academy of Sciences of the United States of America 1986;83:4533-7.

47. Thiruvikraman SV, Guha A, Roboz J, Taubman MB, Nemerson Y, Fallon JT. In situ localization of tissue factor in human atherosclerotic plaques by binding of digoxigenin-labeled factors VIIa and X. Laboratory investigation; a journal of technical methods and pathology 1996;75:451-61.

48. Stojkovic S, Kaun C, Basilio J, et al. Tissue factor is induced by interleukin-33 in human endothelial cells: a new link between coagulation and inflammation. Scientific reports 2016;6:25171.

49. Day SM, Reeve JL, Pedersen B, et al. Macrovascular thrombosis is driven by tissue factor derived primarily from the blood vessel wall. Blood 2005;105:192-8.

50. Pawashe AB, Golino P, Ambrosio G, et al. A monoclonal antibody against rabbit tissue factor inhibits thrombus formation in stenotic injured rabbit carotid arteries. Circulation research 1994;74:56-63.

51. Matetzky S, Tani S, Kangavari S, et al. Smoking increases tissue factor expression in atherosclerotic plaques: implications for plaque thrombogenicity. Circulation 2000;102:602-4.

52. Felmeden DC, Spencer CG, Chung NA, et al. Relation of thrombogenesis in systemic hypertension to angiogenesis and endothelial damage/dysfunction (a substudy of the Anglo-Scandinavian Cardiac Outcomes Trial [ASCOT]). The American journal of cardiology 2003;92:400-5.

53. Lim HS, Blann AD, Lip GY. Soluble CD40 ligand, soluble P-selectin, interleukin-6, and tissue factor in diabetes mellitus: relationships to cardiovascular disease and risk factor intervention. Circulation 2004;109:2524-8.

54. Sambola A, Osende J, Hathcock J, et al. Role of risk factors in the modulation of tissue factor activity and blood thrombogenicity. Circulation 2003;107:973-7.

55. Suefuji H, Ogawa H, Yasue H, et al. Increased plasma tissue factor levels in acute myocardial infarction. American heart journal 1997;134:253-9.

56. Kirkby NS, Lundberg MH, Chan MV, et al. Blockade of the purinergic P2Y12 receptor greatly increases the platelet inhibitory actions of nitric oxide. Proceedings of the National Academy of Sciences of the United States of America 2013;110:15782-7.

57. Bredt DS, Snyder SH. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proceedings of the National Academy of Sciences of the United States of America 1990;87:682-5.

58. Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. Circulation 2004;109:III27-32.

59. Reiner MF, Stivala S, Akhmedov A, et al. Cell-specific off-target effects of ticagrelor but not clopidogrel-active metabolite in endothelial dysfunction. European heart journal 2014;35:199-.

60. Nanhwan MK, Ling S, Kodakandla M, Nylander S, Ye Y, Birnbaum Y. Chronic treatment with ticagrelor limits myocardial infarct size: an adenosine and cyclooxygenase-2-dependent effect. Arteriosclerosis, thrombosis, and vascular biology 2014;34:2078-85.

61. Connolly S, Pogue J, Hart R, et al. Clopidogrel plus aspirin versus oral anticoagulation for atrial fibrillation in the Atrial fibrillation Clopidogrel Trial with Irbesartan for prevention of Vascular Events (ACTIVE W): a randomised controlled trial. Lancet 2006;367:1903-12.

62. Mega JL, Braunwald E, Wiviott SD, et al. Rivaroxaban in patients with a recent acute coronary syndrome. The New England journal of medicine 2012;366:9-19.

63. Dewilde WJ, Oirbans T, Verheugt FW, et al. Use of clopidogrel with or without aspirin in patients taking oral anticoagulant therapy and undergoing percutaneous coronary intervention: an open-label, randomised, controlled trial. Lancet 2013;381:1107-15.

64. Fox KA. Dual or single antiplatelet therapy with anticoagulation? Lancet 2013;381:1080-1.

11 Declaration of personal contributions to work

11.1 Ticagrelor, but not Clopidogrel, Reduces Arterial Thrombosis via Endothelial Tissue Factor Suppression

Cell culture experiments, western blotting, ELISA, data analysis and interpretation in Fig. 1 were done by myself.

Cell culture experiments, data analysis and interpretation in Fig. 2A and B were done by myself, rt-PCR was performed by Alexander Akhmedov, PhD.

Cell culture experiments, western blotting, data analysis and interpretation in Fig. 3A and B were done by myself.

Cell culture experiments, western blotting, data analysis and interpretation in Fig. 4A and B were done by myself, rt-PCR in Fig. 4A was performed by Alexander Akhmedov, PhD.

Cell culture experiments, western blotting, data analysis and interpretation in Fig. 5A and C-D were done by myself. Cell culture experiments, data analysis and interpretation in Fig. 5B were done by myself, rt-PCR was performed by Nicole Bonetti, MD.

Platelet isolation and aggregometry, data analysis and interpretation in Fig. 6A was done by myself. Measurement of ticagrelor plasma concentrations in Fig. 6B was performed by AstraZeneca, Mölndal, Sweden. Surgical procedure, data analysis and interpretation in Fig. 6C was performed by myself. Endothelial TF staining in Fig. 6D was performed by Sophistolab AG, Muttenz, Switzerland; data analysis and interpretation was done by myself. Plasma TF measurement by ELISA in Fig. 6E and endogenous thrombin potential in Fig. 6F as well data analysis and interpretation was done by myself.

Cell culture experiments, western blotting, data analysis and interpretation in Supplemental figure 1 were done by myself.

11.2 Ticagrelor, but not Clopidogrel Active Metabolite, Displays Antithrombotic Properties in the Left Atrial Endocardium

Endocardial cell isolation from atrial appendages of atrial fibrillation patients was performed by Heidi Amstalden, MSc and Martina Glanzmann MSc. Cell culture experiments, western blotting and data analysis in Fig. 1A, B, D and E was performed by Heidi Amstalden, MSc, Martina Glanzmann MSc and myself, data interpretation was done by myself. TF and PAI-1 activity in Fig. 1C and F, respectively, data analysis and interpretation was done by myself. Fig. 1 G was designed by PD Dr. med. Jan Steffel.

Cell culture experiments, western blotting, data analysis and interpretation in Supplemental figure 1 were done by myself.

12 Curriculum Vitae

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Current career

Resident at the Department of Internal Medicine, Cantonal Hospital Baden, Baden, Switzerland (12/2015 – to date) and

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Education and degrees

2014 – 2016	Dr. med. (M.D.), Medical University Zurich, Switzerland Dietary omega-3 alpha-linolenic acid does not prevent venous thrombosis in mice
2006 – 2012	Dr. med. univ. (M.D.), Medical University Innsbruck, Austria Cardiac Morphology and Function in Migfilin-Deficient Mice due to Experimental Pressure Overload
2005 – 2006	Military Service as Paramedic (8 months), Innsbruck, Austria
2000 – 2005	Commercial high school Innsbruck, Austria

Reviewer for scientific journals and institutions

European Heart Journal, Thrombosis Research, Nutrients, Frontiers Physiology, Molecules

Awards and prizes

04/2017 Best Thesis Award 2017, Medical University of Zurich, Switzerland

Dietary omega-3 alpha-linolenic acid does not prevent venous thrombosis in mice

06/2016 Best Abstract in Cardiovascular Biology, Swiss Society of Cardiology

Reiner MF, Diaz-Cañestro C, Akhmedov A, Amstalden H, Briand S, Semerano A, Giacalone G, Keller S, Kullak-Ublick GA, Sessa M, Lüscher TF, Beer JH, Camici GG. Silencing of the Activated Protein-1 Transcription Factor JunD Exacerbates Ischemia/Reperfusion-induced Cerebral Injury.

Cardiovascular Medicine 2016; 19 (Suppl 26)

08/2015 Travel Grant, European Society of Cardiology

06/2014 Young Investigator Award, 60th Annual Meeting of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis

Reiner MF, Stivala S, Lüscher TF, Camici GG, Xiu-Fen M, Yang Z, Beer JH. Arginase II KO reduces platelet aggregation while sparing coagulation in aged mice. *J Thromb Haemost. 2014, Suppl 1:1-106*

Invited lectures

05/2015 Viel Fett, viel Salz: Ist Käse gesund? (A lot of fat and salt: Is cheese healthy?)

Nutrition, Bregenz, Austria

09/2014 Cheese: impacting clinical outcomes by modulation of dietary lipids

European Society for Clinical Nutrition and Metabolism, Geneva, Switzerland

Grants

2017 47'500 CHF, Foundation Kardio Baden (Switzerland), co-applicant

2014 20’000 CHF, Hartmann Müller-Foundation for Medical Research, main applicant

Personal skills and language

German: first language

English: C2

French: A2

Zurich, 20 September 2017

List of publications

Original articles (O)

O13 Akhmedov A, Camici GG, **Reiner MF**, Bonetti N, Costantino S, Holy EW, Spescha RD, Stivala S, Schaub Clerigué A, Speer T, Breitenstein A, Manz J, Lohmann C, Paneni F, Beer JH, Lüscher TF.

Endothelial LOX-1 Activation Differentially Regulates Arterial Thrombus Formation Depending on oxLDL Levels: Role of the Oct-1/SIRT1 and ERK1/2 Pathways.

Cardiovasc Res. 2017 Apr 1;113(5):498-507.

O12 **Reiner MF**, Breitenstein A, Holy EW, Glanzmann M, Amstalden H, Stämpfli SF, Bonetti NR, Falk V, Keller S, Savarese G, Benussi S, Maisano F, Lüscher TF, Beer JH, Steffel J, Camici GG.

Ticagrelor, but not clopidogrel active metabolite, displays antithrombotic properties in the left atrial endocardium.

Eur Heart J. 2017;38(12):916-919.

O11 **Reiner MF**, Akhmedov A, Stivala S, Keller S, Gaul DS, Bonetti NR, Savarese G, Glanzmann M, Zhu C, Ruf W, Yang Z, Matter CM, Lüscher TF, Camici GG, Beer JH. Ticagrelor, but not clopidogrel, reduces arterial thrombosis via endothelial tissue factor suppression.

Cardiovasc Res. 2017 Jan;113(1):61-69.

O10 **Reiner MF**, Stivala S, Limacher A, Bonetti NR, Méan M, Egloff M, Rodondi N, Aujesky D, von Schacky C, Lüscher TF, Camici GG, Beer JH.

Omega-3 Fatty Acids Predict Recurrent Venous Thromboembolism or Total Mortality in Elderly Patients with Acute Venous Thromboembolism.

J Thromb Haemost. 2017 Jan;15(1):47-56.

O9 Breitenstein A, Stämpfli SF, **Reiner MF**, Shi Y, Keller S, Akhmedov A, Schaub Clerigué A, Spescha RD, Beer HJ, Lüscher TF, Tanner FC, Camici GG.

The MAP kinase JNK2 mediates cigarette smoke-induced arterial thrombosis.

Thromb Haemost. 2017 Jan 5;117(1):83-89.

O8 Spescha RD, Klohs J, Semerano A, Giacalone G, Derungs RS, **Reiner MF**, Rodriguez Gutierrez D, Mendez-Carmona N, Glanzmann M, Savarese G, Kränkel N, Akhmedov A, Keller S,

Mocharla P, Kaufmann MR, Wenger RH, Vogel J, Kulic L, Nitsch RM, Beer JH, Peruzzotti-Jametti L, Sessa M, Lüscher TF, Camici GG.

Post-ischaemic silencing of p66Shc reduces ischaemia/reperfusion brain injury and its expression correlates to clinical outcome in stroke.

Eur Heart J. 2015 Jul 1;36(25):1590-600.

O7 Haubner BJ, Moik D, Schuetz T, **Reiner MF**, Voelkl JG, Streil K, Bader K, Zhao L, Scheu C, Mair J, Pachinger O, Metzler B.

In Vivo Cardiac Role of Migfilin during Experimental Pressure Overload.

Cardiovasc Res. 2015 Jun 1;106(3):398-407.

O6 Savarese G, Rosano GM, Parente A, D'Amore C, **Reiner MF**, Camici GG, Trimarco B, Perrone-Filardi P.

Reduction of C-reactive protein is not associated with reduced cardiovascular risk and mortality in patients treated with statins. A meta-analysis of 22 randomized trials.

Int J Cardiol. *2014 Nov 15;177(1):152-160.*

O5 Akhmedov A, Montecucco F, Braunersreuther V, Camici GG, Jakob P, **Reiner MF**, Glanzmann M, Burger F, Paneni F, Galan K, Pelli G, Vuilleumier N, Belin A, Vallée JP, Mach F, Lüscher TF.

Genetic deletion of the adaptor protein p66Shc increases susceptibility to short-term ischaemic myocardial injury via intracellular salvage pathways.

Eur Heart J. 2015 Feb 21;36(8):516-26a.

O4 **Reiner MF**, Martinod K, Stivala S, Savarese G, Camici GG, Lüscher TF, Wagner DD, Beer JH.

Dietary omega-3 alpha-linolenic acid does not prevent venous thrombosis in mice.

Thromb Haemost. 2015 Jan 8;113(1):177-84.

O3 Holy EW, Besler C, **Reiner MF**, Camici GG, Manz J, Beer JH, Lüscher TF, Landmesser U, Tanner FC.

High-density lipoprotein from patients with coronary heart disease loses anti-thrombotic effects on endothelial cells: impact on arterial thrombus formation.

Thromb Haemost. 2014 Nov 3;112(5):1024-35

O2 Savarese G, Dei Cas A, Rosano G, D'Amore C, Musella F, Mosca S, **Reiner MF**, Marchioli R, Trimarco B, Perrone-Filardi P.

Reduction of albumin urinary excretion is associated with reduced cardiovascular events in hypertensive and/or diabetic patients. A meta-regression analysis of 32 randomized trials.

Int J Cardiol. 2014 Mar 15;172(2):403-10.

O1 Stivala S, **Reiner MF**, Lohmann C, Lüscher TF, Matter CM, Beer JH.

Dietary α-linolenic acid increases the platelet count in ApoE-/- mice by reducing clearance.

Blood. 2013 Aug 8;122(6):1026-33.

Reviews (R)

R3 Reiner MF, Stivala S, Beer JH.

Alpine cheese in cardiovascular disease.

Eur Heart J. 2015 Aug 14;36(31):2023-2030. CardioPulse Articles.

R2 Reiner MF, Stivala S, Camici GG, Beer JH.

The effects of Omega-3 fatty acids in clinical medicine.

Praxis (Bern 1994). 2014 Mar 12;103(6):329-35.

R1 Martin F. Reiner, Simona Stivala, Jürg H. Beer.

Omega-3-Fettsäuren, Schweizer Alpkäse und deren Auswirkungen auf das kardiovaskuläre System.

Schweiz. Zeitschr. f. Ernährungsmed. 2012;5:1–5.